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IAP Regulation of Tumor Metastasis: A Dissertation

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IAP regulation of tumor metastasis

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Statement of Contribution

The work presented in Chapter 3 was done in collaboration with Takehiko Dohi, Research Assistant Professor in our lab. The mouse splenic surgeries were performed by Christopher Raskett and Takehiko Dohi. I am responsible for all the experiments discussed in Chapter 2. The invasion and migrations assays were taught by Keith Merdek, a former postdoctoral associate in Dr. Arthur Mercurio's lab. I have acknowledged all the individuals who have shared their reagents in the Materials and Methods section.

Abstract

The dissemination of tumor cells to distant organs i.e. metastasis is an exceedingly complex process leading to 90% of all cancer deaths. Despite being so clinically important, little is known about this process that requires tumor cells to leave the primary tumor site, intravasate and transport through the blood stream, extravasate and colonize at secondary sites leading to distant metastases. Survivin, a member of the IAP (Inhibitor of Apoptosis) family with known functions in apoptosis and mitosis, is highly expressed in aggressive tumors and is associated with poor prognosis and adverse clinical outcome. But the mechanistic role of survivin in metastatic dissemination has not been investigated. In this study, we demonstrate an important and novel role of survivin in activating a broad gene expression program in tumor cells. Of particular importance is the upregulation of a distinct class of cell adhesion molecules, particularly fibronectin. This IAP mediated gene regulation requires synergistic intermolecular cooperation between survivin and its related cofactor molecule, XIAP that results in activation of NF-κB dependent fibronectin gene expression. The binding of fibronectin with its cognate cell surface receptors initiates outside–in signaling leading to the autocrine and paracrine activation of cell motility kinases, FAK and Src, in turn leading to enhanced tumor invasion and metastasis. The importance of survivin and XIAP in the process of metastasis has also been demonstrated *in vivo* using intrasplenic injections in mouse models.

Overall this study is the first to place survivin upstream of transcriptional activation of gene expression particularly fibronectin. In addition, it also demonstrates the

importance of survivin-XIAP complex in mediating NF-κB activation which in turn switches on the expression of various target genes involved in tumor metastasis. Hence this study dissects the upstream and downstream requirements of survivin- XIAP complex mediated tumor dissemination and metastasis.

Significance of this Study

The hallmark of end-stage cancer is metastasis, an incurable condition almost invariably associated with death from disease. Despite a better understanding of the metastatic process, and the identification of key gene expression requirements of this pathway, the development of anti-metastatic therapies has lagged behind, with no viable options being currently offered in the clinical setting. Our findings that Inhibitor of Apoptosis (IAP) proteins functions as metastasis-promoting genes independently of cell survival, but through activation of cell motility could have important ramifications for the broader application of IAP antagonists currently in early clinical trials, as novel anti-metastatic therapies.

Table of Contents

Table of Figures

List of Tables

List of Abbreviations

ASAP1	Arf GTPase-activating protein
BIR	Baculovirus IAP repeat, conserved motif of $CX_2CX_{16}HX_{6-8}C$
BMDC	Bone marrow derived cells
CARD	Caspase recruitment domain
CAS	Cellular apoptosis susceptibility
CDK1	Cyclin dependent kinase1
CRM1	chromosome region maintenance protein 1
CXCR4	Chemokine (CXC motif) receptor 4
cDNA	Cloned DNA, coding region of organism's DNA
DAPI	4',6-diamidino-2-phenylindole
DBLCL	Diffuse large B-cell lymphoma
DMSO	Dimethyl sulfoxide, polar aprotic solvent
ECM	Extracellular matrix protein
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor, also known as ErbB1
EMSA	Electrophoretic mobility shift assay
EMT	Epithelial mesenchymal transition
ERK	Extracellular signal regulated protein kinase pathway
FAK	Focal Adhesion kinase
FasL	FAS ligand
FAT	Focal adhesion targeting
FERM	F for Band 4.1, E for Ezrin, R for Radixin, M for Moesin
FGF	Fibroblast growth factor
FN	Fibronectin
FRNK	FAK related non-kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GTP	Guanosine-5'-triphosphate
HBXIP	Hepatitis B-X-interacting protein
HGF	Hepatocyte Growth Factor
HPC	Hematopoetic precursor cells
HSP90	Heat shock protein 90
IAP	Inhibitor of apoptosis protein
ICAM-1	Inter-Cellular Adhesion Molecule 1
IgG	Immunoglobulin
IKK	IκB kinase

INCENP	Inner centromere protein
JNK	c-Jun N-Terminal Kinase
MAPK	Mitogen activated protein kinase
MEF	Mouse embyonic fibroblasts
MEK	Erk/MAP kinases
MEN	Mitotic exit network
MMP	Matrix metalloproteinases
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide,
NFκB	Nuclear factor of κ light polypeptide gene enhancer in B-cells
NIK	NF-kappa B-inducing kinase
NK	Natural Killer
NSCLC	Non Small cell lung cancer
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PKA	Cyclic AMP-dependent protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PyMT	Polyoma middle T oncoprotein
RLU	Relative Luciferase Units
RNA	Ribonucleic acid
RING	Really interesting new gene, zinc finger motif
ROS	Reactive oxygen species
SCID	Severe combined immunodeficiency
SDF-1	Stromal derived factor
TAB1	Transforming growth factor-β-activated protein kinase 1-binding protein 1
TAK1	Transforming growth factor-alpha-activated kinase 1
TBS	Tris buffered Saline
TNF	Tumor Necrosis factor
TNFR	Tumor Necrosis factor receptor
VCAM-1	Vascular cell adhesion molecule-1
VEGFR	Vascular endothelial growth factor receptor
VLA-4	Very Late Antigen-4, another name for Integrin α4β1
XIAP	X-linked inhibitor of Apoptosis

Chapter 1: Introduction

The dissemination of tumor cells to distant organs, i.e. metastasis (Nguyen and Massague, 2007), heralds a nearly invariably fatal phase of epithelial malignancies, with few, if any, therapeutic options. Tumor metastasis is a highly complex and dynamic event that requires tumor cells to dissociate from the primary tumor mass and move and localize to distant or secondary sites (Chambers et al., 2001; Woodhouse et al., 1997). The essential steps in the metastatic cascade include invasion of the adjacent tissues, intravasation, survival in circulation when detached from the extracellular matrix, a condition called anoikis (Frisch and Screaton, 2001), transport in blood and lymphatic vessels, extravasation, colonization and growth at distant sites with unrelated microenvironments (Weigelt et al., 2005). To successfully complete the 'metastatic journey' cancer cells acquire distinct mechanisms and activate several gene expression programs and hence several models of metastasis have been proposed (Nguyen et al., 2009). One of the models suggests that metastatic ability is an acquired event wherein the primary tumor cells acquire genetic alterations during the course of time and become more invasive and metastatic (Scheel et al., 2007). The other model proposes that the metastatic capacity of primary tumors is pre-determined at the onset of tumorigenesis and gene expression signatures exist which can distinguish localized tumors from tumors that metastasize to distant locations (Talmadge, 2007). Despite opposing views, one invariable feature of the metastatic process is deregulated gene expression (Nguyen and Massague, 2007). Genes that promote metastasis have been categorized into three distinct

classes based on their level of participation (Nguyen and Massague, 2007). They include *metastasis -initiation genes* that promote tumor invasion, cell motility and angiogenesis. The gain or loss of function of these genes allows tumor cells to invade the basement membrane and escape into the circulation. The second class of genes is referred to as *metastasis-progression genes* that confer a specific organ tropism. They are involved in functions such as vascular remodeling and provide the malignant cells the ability to infiltrate the distant organs. They could be enriched at the primary site but possibly have a unique role at the distant site. The third class is called *metastasis-virulence genes* that promote growth and colonization at distant sites. They do not affect the primary growth but provide selective advantage at the secondary site (Nguyen and Massague, 2007). Metastasis suppressors such as caspase 8 have also been strongly implicated in tumor metastasis (Stupack et al., 2006)

Section I: Steps of Metastasis

1. Tumor Migration and Invasion

Tumor invasion is the initial event in the metastatic cascade and is primarily mediated by regulated interaction of tumor cells with the surrounding extracellular matrix (ECM). This step of metastasis requires tumor cells to attach, proteolyze and migrate through the basement membrane and enter into the circulation (Fidler et al., 1978). The extracellular matrix is composed of both interstitial component and the basement membrane. The extracellular-matrix components, such as fibronectin, collagen, and Laminin, act as a scaffold allowing cells to attach and move by making contacts with the cell-surface receptors called integrins (Chiang and Massague, 2008). As one of the most abundant

constituents of the extracellular matrix, fibronectin binds multiple integrins (Cukierman et al., 2002), and this binding results in the activation of focal adhesion kinase (FAK) (Sieg et al., 2000), Src (Yeatman, 2004), Akt (Irie et al., 2005), as well as modulation of the small GTPases of the Rho family (Nobes and Hall, 1995). In response to these signals, cells remodel their actin cytoskeleton (Juliano et al., 2004), express matrix metalloproteinases (Han et al., 2006), become migratory (Livant et al., 2000), invade basement membranes (Gaggioli et al., 2007), and acquire the ability to resist apoptosis (Fornaro et al., 2003). Increased fibronectin expression has been shown in various tumor cell types like melanomas (Bittner et al., 2000), breast cancers (Jiang et al., 2002), and thyroid carcinomas (Huang et al., 2001c). Global gene expression profiling has shown that increased fibronectin expression leads to oncogenic transformation of melanocytes (Bittner et al., 2000) and tumor-derived fibronectin help melanoma cells to become more invasive and metastatic (Gaggioli et al., 2007). Apart from its role in tumor invasion, fibronectin has been shown more recently to play an important role in the colonization of primary tumor cells at the distant sites by forming a 'premetastatic niche' (Kaplan et al., 2006).

Integrins are heterodimeric transmembrane proteins containing 1 of 18 α- and 1of 8 β subunits. The different α- and β chain combinations dictate the specificity for different ECM components (Hynes, 2002). For example, $\alpha 5\beta 1$ selectively binds fibronectin whereas $\alpha v\beta 3$ binds to fibronectin, vitronectin, Von Willebrand factor and cleaved forms of collagen and laminin (van der Flier and Sonnenberg, 2001). A number of studies have demonstrated that more migratory and invasive cells show dramatic

alterations in the levels of integrin and ECM expression. For example αvβ3 integrin overexpression is seen at the invasive front of malignant melanoma cells (Brooks et al., 1994). Similarly α6β4 has been implicated in the invasion of colorectal carcinoma cells (Chao et al., 1996).

Integrins form a functional link between the ECM and the intracellular signaling pathways that influence cell shape, motility, proliferation and migration and invasion (Aplin et al., 1999). 'Outside–in signaling' mediated by binding of ECM (Juliano et al., 2004) to integrins leads to the clustering of integrins that initiates several downstream signaling events. Since integrins lack intrinsic catalytic activity, signals initiated by ECM–integrin interactions are transduced inside the cells through activation of some non-receptor tyrosine kinases like focal adhesion kinase (FAK) and Src.

Focal Adhesion Kinase (FAK)

FAK is a 120 Kilodalton cytoplasmic protein tyrosine kinase that co-localizes with integrins to structures referred to as 'focal contacts'(Parsons, 2003). Structurally, FAK contains several distinct domains (Schaller, 2001). It contains a central catalytic domain flanked by an amino-terminal FERM domain and a carboxy terminal proline-rich and FAK targeting domain (FAT) (Fig 1-2). The FERM domain is a key regulatory element as it interacts with several proteins including cytoplasmic tails of integrins (Calderwood et al., 1999), integrins associated proteins (Mitra and Schlaepfer, 2006) and epidermal growth factor receptor (EGFR) (Sieg et al., 2000). The FAT domain at the carboxy terminus is important in FAK localization by interacting with focal adhesion protein, paxillin (Hayashi et al., 2002), whereas the proline rich domains are involved in

binding with proteins containing SH3 domains including p130 Cas (Harte et al., 1996), GRAF (Taylor et al., 1998) and ASAP1 (Liu et al., 2002). FAK is maintained in an inactive state by binding of its FERM domain to the kinase domain (Lietha et al., 2007). Integrin clustering leads to intracellular signals that relieve the autoinhibition, resulting in stimulation of FAK catalytic activity by autophosphorylation at tyrosine 397 (Y397). This auto-phosphorylation event creates a high-affinity binding site for the SH2 domain of Src family kinases and leading to the activation of Src (Schaller et al., 1994; Xing et al., 1994). The activated Src in turn phosphorylates additional tyrosine residues on the kinase domain (Y576 and Y577) and carboxy-terminal domain (Y861 and Y925) of FAK. These phosphorylated residues form docking sites for other cytoplasmic proteins including p130 Cas and paxillin (Schaller et al., 1999), p21-activated kinase and mitogen-activated protein kinase (MAPK) pathways and the small GTPases, Rac and Rho leading to the activation of various signaling cascades involved in migration and invasion (Schlaepfer and Mitra, 2004). In normal cells, FAK activity is under tight regulation by several mechanisms including gene amplification, alternative gene splicing (Toutant et al., 2002)and action of phosphatases (Shen and Schaller, 1999). On the contrary, FAK levels are dramatically altered in a wide range of malignancies. The levels are increased in tumors of prostate (Tremblay et al., 1996), ovary (Judson et al., 1999), colon and breast (Cance et al., 2000). Increased FAK expression and activity is closely linked to metastasis and poor prognosis (Cance et al., 2000; Owens et al., 1995; Schlaepfer and Mitra, 2004). They are expressed at higher levels in invasive tumors as compared to benign preneoplastic lesions (Owens et al., 1995).

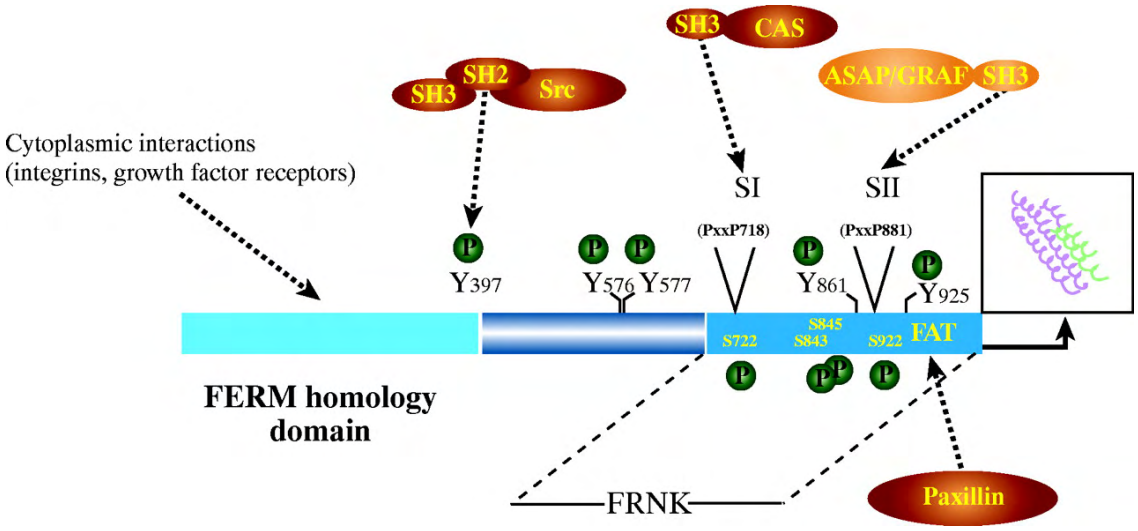

Figure 1-1: Organization of the domains of focal adhesion kinase (FAK)

(Parsons, 2003)

FAK deletion in a mouse skin tumorigenesis model has been shown to decrease papilloma formation and block progression to malignant squamous cell carcinoma (McLean et al., 2004). Phosphorylation of FAK at Y-397 residue has also been implicated in more aggressive tumors. In ovarian tissues, enhanced expression of phosphorylated FAK Y-397 was seen in invasive tumors as compared to the normal epithelium. The inhibition of FAK activity using a dominant negative form of FAK called FRNK (FAK related non-kinase) leads to the inhibition of cell migration, invasion and proliferation ((Hauck et al., 2002; Hauck et al., 2001; Slack et al., 2001). A study by Van de water (van Nimwegen et al., 2005) showed that inducible expression of FRNK in mammary adenocarcinoma cells suppressed tumor growth and lung metastasis significantly. Recently, a conditional knockout study of FAK deletion showed that abolishing FAK reduced Polyoma middle T oncoprotein (PyMT) induced breast tumorigenesis and reduced the ability of mammary tumor cells to invade and metastasize to lung (Pylayeva et al., 2009). Additionally, numerous studies using antisense and dominant negative mutants have strongly correlated the role of FAK in mediating cell migration and invasion of tumor cells. These data are summarized in Table 1- 1.

FAK has also been implicated in deregulation of E-cadherin expression, a protein involved in epithelial mesenchymal transition (EMT). This is another prominent mechanism involved in tumor cell invasion. During this process epithelial cells lose the cell-cell contacts and epithelial markers like E-cadherin and γ-catenin and acquire fibroblast like phenotype and mesenchymal markers like fibronectin, vimentin, N-cadherin. This switch from epithelial to mesenchymal phenotype is important for most

Cells	FAK status	Biological Effect	Reference
Melanoma	Inhibit expression with antisense or FRNK	Loss of cell adhesion	(Maung et al., 1999)
Melanoma (highly metastatic)	Inhibit signaling with FRNK	Inhibit migration Inhibit invasion	(Li et al., 2001c)
Breast	Inhibit expression with antisense	Irreversible loss of adhesion	(Xu et al., 2000; Xu et al., 1998)
NSCLC	Inhibit expression with antisense	Decrease adhesion independent growth	(Leyton et al., 2001)
Prostate carcinoma	Inhibit signaling with FRNK	Inhibit migration	(Slack et al., 2001)
Ovarian cancer	Inhibit expression with antisense	Inhibit invasion	(Shibata et al., 1998)
Adenocarcinoma	Inhibit expression with antisense, no expression (FAK-/-), inhibit signaling with FRNK	Inhibit EGF-mediated motility and invasion	(Hauck et al., 2001)
Astrocytoma	Increase expression by overexpression	Increase migration	(Zagzag et al., 2000)

Table 1-1: Evidence showing correlation of FAK expression and activity in cell migration and invasion of tumor cells
Modified from (Gabarra-Niecko et al., 2003)

tumors to become invasive and aggressive (Kang and Massague, 2004). Some of the transcription factors mediating EMT and involved in metastatic dissemination are TWIST1 (Yang et al., 2004), Snail (Barrallo-Gimeno and Nieto, 2005) and Slug (Alves et al., 2009).

Src family of Kinases (SFKs)

The other family of non-receptor tyrosine kinases is the Src family of kinases. They are associated with FAK and integrins at focal contacts and play an important role in the integrin signaling. This family includes c-Src, Fyn, Yes, Blk, Lyn, Hck and Lck (Thomas and Brugge, 1997). c-Src is the most extensively studied member of this family and has been implicated in tumor progression (Irby and Yeatman, 2000). Discovered by Michael Bishop and Harold Varmus, c-Src is the first cellular homologue of the retroviral v-Src protein identified in the late 1970s by Francis Peyton Rous(Stehelin et al., 1977) . c-Src and v-Src share similar structures except for a truncation in the regulatory carboxy terminus which features loss of the auto-inhibitory tyrosine 527 residue (Takeya and Hanafusa, 1982) making v-Src constitutively active. Unlike v-Src, c-Src is non-transforming in nature but once activated, can be converted to a transforming protein by various modifications including dephosphorylation of tyrosine-527 which is consistent with its role as a proto-oncogene (Der, 1987). Src protein is characterized by several distinct domains. It contains C-terminal domain containing a negative regulatory tyrosine residue (Tyr-527), four SH (Src homology) domains and a N-terminal domain which may undergo myristoylation (Engen et al., 2008). Src is present in an inactive state and inhibited state under normal conditions (Brown and Cooper, 1996). Binding of the of Src-

SH2 domain to FAK brings about a conformational change in Src relieving the auto inhibition of Src kinase and phosphorylation of the activating tyrosine residue (Tyr-416) on the first SH1 kinase domain of Src (Schlaepfer et al., 2004) . The activated Src and FAK form a functional bipartite kinase complex leading to the activation of several downstream signaling cascades important in cell motility, adhesion and invasion (Mitra and Schlaepfer, 2006). FAK-Src signaling has been shown to be involved in activation of Rac1 activity (Brown et al., 2005; Ren et al., 2000), a member of the Rho family of GTPases involved in cytoskeletal remodeling (Raftopoulou and Hall, 2004). FAK-Src signaling has also been implicated in the transcriptional activation of MMP-2 and MMP-9 (Hsia et al., 2003). Overexpression of c-Src and an increase in its catalytic activity has been implicated in various types of cancers (Irby and Yeatman, 2000) and increased c-Src kinase activity has been implicated with enhanced metastatic potential (Mao et al., 1997; Talamonti et al., 1993) through effects on motility and invasion (Jones et al., 2002).

2. Survival in circulation-Anoikis Resistance

Once tumor cells detach from the primary tumor mass, they enter into the circulatory or lymphatic system. This is called intravasastion. Once in the circulation, they no longer get the survival signals they normally receive from cell-cell and cell-ECM interactions. In normal epithelial cells the lack of cell-ECM interactions activates a special form of cell death called ‘anoikis’(Greek word for homelessness) (Frisch, 2000).This is a safeguarding mechanism for normal cells to maintain tissue homeostasis and development and has significant physiological relevance. Various studies have focused on the importance of anoikis in the process of neoplastic transformation as seen

in malignant mammary (Streuli and Gilmore, 1999)and colon cancers (Shanmugathasan and Jothy, 2000). Tumor cells with metastatic potential acquire several mechanisms to evade this process and acquire resistance to anoikis which not only provides them with increased survival time but also facilitates reattachment and colonization at secondary sites. The molecular mechanisms involved in anoikis resistance are still not completely understood. Some of the prominent mechanisms include activation of survival pathways (PI3 kinase-AKT), upregulation of matrix metalloproteinases , inactivation of p53, overexpression of focal adhesion kinase (FAK) and anti-apoptotic proteins (BCL-2, BCL-XL,XIAP) (Liotta and Kohn, 2004; Mehlen and Puisieux, 2006; Townson et al., 2003). A summary of the relevant pathways involved in anoikis resistance is shown in Table 1.2.

3. Colonization and Growth at Distant Sites

The last and final stage of the metastatic cascade is the colonization of the secondary site. Stephen Paget proposed a ‘seed and soil’ hypothesis according to which the disseminated tumor cells (seed) colonize to specific organs whose microenvironment (soil) is compatible for their growth and proliferation (Paget, 1889) For example, breast cancers frequently metastasize to lungs, liver, bone and brain whereas prostate cancers colonize to bone. This hypothesis was refuted by James Ewing who proposed that distant metastasis at specific sites is primarily determined by the vasculature at the primary site (Ewing, 1928). Recent advances and emerging data strongly support Paget’s hypothesis and stresses on the concept of viable tumor microenvironment or ‘premetastatic niche’ in

Mechanisms involved in anoikis resistance of tumor cells	Activated pathways	References
Constitutive activation of survival signals (PI3K, MEK/ERK, and NF-κB)	- Melanoma cells – Acquisition of autocrine loops for bFGF, HGF, PDGF-AA mediate proliferation,survival and migration. -Ovarian Cancer- upregulation of neurotrophin receptor,TrkB and subsequent activation of PI3K/Akt pathway lead to anoikis resistance	(Nesbit et al., 1999) (Li et al., 2003) (Douma et al., 2004)
Altered integrin mediated signaling 1. Increased FAK activation 2. Increased Src activation 3. Changes in pattern of integrin expression	1.Inhibition of FAK activity leads to anoikis in lung adenocrcinoma cells, A549 2. Src activation mediates anoikis resistance in colon, Lung adenocarcinoma and osteosarcoma cells 3.Melanoma cells: de novo expression of αvβ3 integrin allows for survival within the dermis	(Liu et al., 2008) (Windham et al., 2002) (Montgomery et al., 1994) (Petitclerc et al., 1999)
Hypoxia	- Hypoxia-driven N-cadherin expression promote anoikis resistance by induction of PKB/Akt activation and Bad inhibition - Hypoxia-induced ROS increase could be responsible for down-regulation of pro-apoptotic molecules	(Li et al., 2001a) (Chandel et al., 1998)
Apoptosis regulators	Activation of antiapoptotic members like Bcl2 and XIAP implicated in promoting anoikis resistance	(Berezovskaya et al., 2005; Galante et al., 2008)
Epithelial mesenchyman transition (EMT)	Twist, Snail, and NF-κB , transcription factors involved in the acquisition of anoikis resistance during the EMT process	(Kucharczak et al., 2003; Vega et al., 2004; Yang et al., 2004)

Table 1-2: Mechanisms and pathways involved in anoikis resistance of tumor cells
Modified from (Chiarugi and Giannoni, 2008)

grafting primary tumor cells to distant sites (Kaplan et al., 2006). Mobilization of bone marrow derived hematopoietic progenitor cells (HPCs) to the distant sites have been shown to be involved in metastatic initiation (Kaplan et al., 2005) by providing permissive tumor microenvironment at distant sites. These HPCs have been shown to express VEGFR1 and other progenitor markers like CD34, CD11b, c-kit, and Sca-1 and hence maintain their progenitor status at metastatic sites. Inhibition of VEGFR1 by neutralizing antibodies completely prevented metastasis. The bone marrow derived cells (BMDC) are tumor type specific as when LLC (Lewis Lung carcinoma cells are intradermally injected in animals, the BMDC cluster formation is seen specifically in lungs. $VEGFR1^{+}$HPCs have been shown to express elevated levels of VLA-4 ($\alpha 4\beta_1$) which binds to the newly synthesized fibronectin produced by fibroblasts in the tissue microenvironment. This allows the BMDC to adhere and form the premetastatic niche. Fibronectin interaction with VLA-4 also regulated the MMP-9 expression (Huhtala et al., 1995). Upregulated MMP-9 expression degraded the basement membrane, and lead to the extravasation of more $VEGFR1^{+}$ cells into the permissible niche. The $VEGFR1^{+}$ HPCs, newly formed fibronectin and associated stromal cells alter the local microenvironment, leading to the activation of chemokines such as SDF-1 which attracts tumor cells expressing SDF-1 receptor CXCR4 as seen in previous studies (Balkwill, 2004; Muller et al., 2001) hence leading to enhanced attachment, survival, growth and retention of tumor cells at distant sites.

Section II: Apoptosis Regulators and Tumor Progression

Aberrantly increased cell survival or resistance to death signals, as often observed in tumors, is an invariable requirement of metastasis (Mehlen and Puisieux, 2006). Apoptosis or programmed cell death is a tightly regulated cell suicide program required for normal cellular development and tissue homeostasis. Apoptosis is mediated by two distinct pathways called extrinsic and intrinsic pathways (Chen and Wang, 2002), both of which mediate apoptosis by forming multimeric complexes, which in turn activate several downstream caspases, key executioners of apoptosis (Bieberich et al., 2004; Cohen, 1997). Extrinsic (death receptor mediated) pathway is initiated by binding of ligands like TNF-α and Fas ligand (FasL) to their receptors TNFR and Fas (Krammer, 2000) leading to activation of caspase 8, one of the initiator caspases. Intrinsic or mitochondria dependent pathway is activated by several intracellular signals like DNA damage, hypoxia and other intracellular cues. This leads to mitochondrial dysfunction (Wang, 2001) leading to release of cytochrome c and Smac. These mitochondrial changes lead to activation of caspase 9, an initiator caspase which then activates the downstream signaling cascade resulting in apoptosis.

Defects in the regulation of apoptosis lead to malignant transformation. Indeed, the evasion of apoptosis has been listed as one of the six hallmarks of cancer (Hanahan and Weinberg, 2000). Since multiple mechanisms function at many levels to mediate apoptosis, a number of opportunities for apoptotic deregulation are available and can be exploited by the tumor cells. One of the several molecular mechanisms by which cancer cells are protected from apoptosis is through deregulated expression of protective

proteins like anti-apoptotic members of the Bcl-2 family or members of the inhibitor of apoptosis (IAP) family implicated in controlling mitochondrial integrity (Cory and Adams, 2002), or suppressing endogenous caspase activity (Eckelman et al., 2006), respectively. Apoptosis deregulation has also been implicated in drug resistance as various chemotherapeutic agents act via activation of apoptosis (Pommier et al., 2004).

Bcl2 family

Bcl2 (B-cell lymphoma 2) family is comprised of both anti-apoptotic proteins that include that include Bcl-2, Bcl-XL, Bcl-w and pro-apoptotic members Bax, Bak, Bad, Bid, Bik. All the members of this family are characterized by the presence of 1-4 copies of BH (Bcl2 homology) domains (Figure1-2) and play an important role in the intrinsic pathway of apoptosis by regulating mitochondrial permeability (Cory and Adams, 2002). Bcl-2, the most well studied gene of this family, was discovered in follicular lymphoma where in Bcl2 was translocated into the immunoglobulin heavy chain locus resulting in upregulation of Bcl2 expression in B cells ((Tsujimoto et al., 1985).

The oncogenic potential of Bcl2 has been demonstrated in various transgenic mouse models (Strasser et al., 1990; Vaux et al., 1988) and is primarily mediated by its ability to inhibit apoptosis without enhancing cell proliferation (Vaux et al., 1988). In fact, paradoxically, it has been shown to be anti proliferative and delays the G0 to S transition (Borner, 1996). The co-existence of contrasting anti apoptotic and anti-proliferative functions of Bcl2 explains why Bcl2 induce tumors with low penetrance and require other genetic alterations like Myc for tumorigenesis (Fanidi et al., 1992; Strasser et al., 1990). Nevertheless, overexpression of Bcl2 has been detected in several human

cancers including breast (Leek et al., 1994), prostate (McDonnell et al., 1992) colon (Hague et al., 1994) and lung (Ikegaki et al., 1994). Stable expression of Bcl2 has been shown to induce increased metastatic potential in pancreatic cell lines (Bold et al., 2001). However, most of the studies show that the effects of Bcl2 on tumor progression are primarily because of its antiapoptotic function which is mediated directly by controlling the activation of caspases (Adams and Cory, 1998) or indirectly by maintenance of the mitochondrial integrity (Green and Reed, 1998). The pancreatic cell line, Mia-Panc-1 stably expressing Bcl2 is more resistant to apoptosis and forms larger primary tumors and higher number of liver metastasis (Bold et al., 2001).

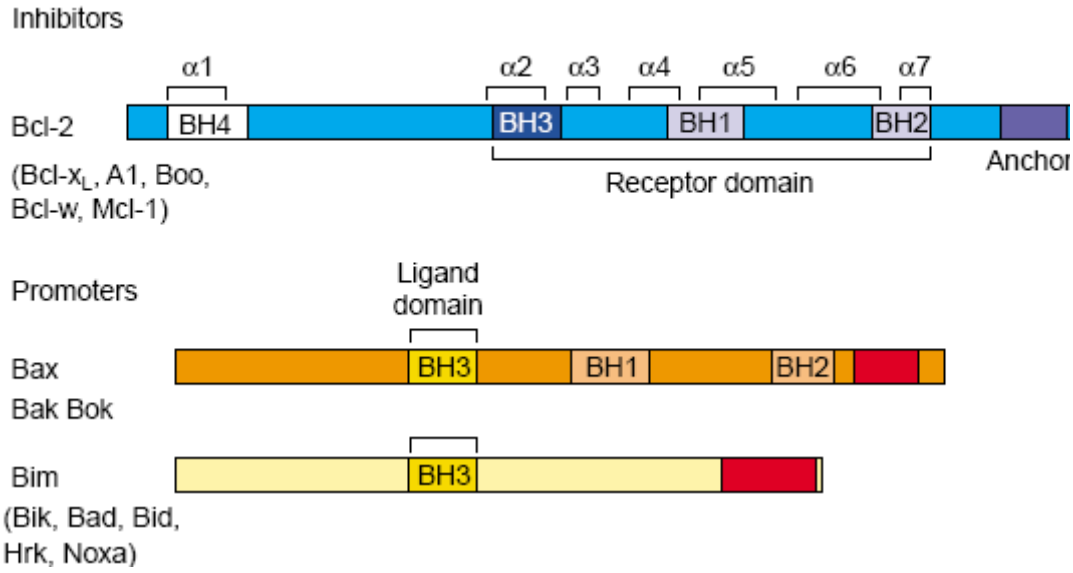

Figure 1-2: Schematic representation of the Bcl2 family of proteins

(Adams and Cory, 2001)

Inhibitor of Apoptosis (IAP) Family

The inhibitor of apoptosis (IAP) gene was discovered 15 years back by Lois Miller and colleagues (Crook et al., 1993). This discovery led to the identification of cellular homologs in different species ranging from lower vertebrates to higher vertebrates . So far, eight members of this family have been identified in humans and are characterized by the presence of one or three baculovirus IAP repeat (BIR) protein domains of approximately 70 amino-acid residues (Hinds et al., 1999; Miller, 1999). Several IAPs also contain a CARD (caspase associated recruitment domain) and a RING domain (Joazeiro and Weissman, 2000) as shown in Figure 1.2. RING domains have been shown to function as E3 ubiquitin ligases. These RING domains work together with ubiquitin activating (E1) and conjugating (E2) enzymes to attach a ubiquitin moiety to the target proteins which then gets degraded by the proteasome (Jesenberger and Jentsch, 2002; Yang et al., 2000). IAP proteins are involved in mediating various diverse and non redundant functions ranging from inhibition of apoptosis, to mitotic regulation. Caspase inhibition is one of the several mechanisms employed by IAPs like X-linked inhibitors of apoptosis (XIAP) to regulate apoptosis. They bind directly to caspase 3 and caspase 7 and inhibit their function. Several caspase independent mechanisms like NF-κB (Hofer-Warbinek et al., 2000) and Mitogen activated protein (MAP) kinase JNK1 (Sanna et al., 2002) activation have also been implicated in IAP mediated apoptotic inhibition. Although named for its role in apoptotic inhibition, IAPs have also been implicated in other diverse roles like copper homeostasis (Burstein et al., 2004), cell division (Srinivasula and Ashwell, 2008), ubiquitination (Park et al., 2008) and cell motility

(Geisbrecht and Montell, 2004). In this study, we have focused on survivin and XIAP, two of the more prominent members of this family that have gained much attention in the past several years.

Structure and Functions of Survivin

Survivin, a 16.5 kilodalton protein is a unique and smallest member of the IAP family (Salvesen and Duckett, 2002). It differs from the other members both structurally and functionally. It contains a single BIR domain (Refer to Figure 1.2), forms stable homodimers in solution (Verdecia et al., 2000), and its expression is regulated in a cell cycle dependent manner with a peak expression at the G2-M phase of the cell cycle (Li et al., 1998). Survivin is an oncofetal protein playing an important role in embryonic development. Survivin knockout mice die prematurely at embryonic day 3.5 with various mitotic and apoptotic defects suggesting an important role of survivin in mitotic regulation and apoptosis (Uren et al., 2000). However, its expression is undetectable in most adult cells and dramatically increased in virtually all human tumors tested suggesting an extremely important role played by survivin in tumorigenesis (Ambrosini et al., 1997) Survivin's role in apoptotic inhibition has been characterized using both *in vivo* and *in vitro* models (Grossman et al., 2001; Kanwar et al., 2001; Olie et al., 2000). Increased expression of survivin has been shown to inhibit both the intrinsic and extrinsic apoptotic pathways and is primarily mediated by its interactions with other adaptor molecules and members of the apoptotic pathway through its N-terminal BIR domain (Ambrosini et al., 1997; Kobayashi et al., 1999; Mahotka et al., 1999).

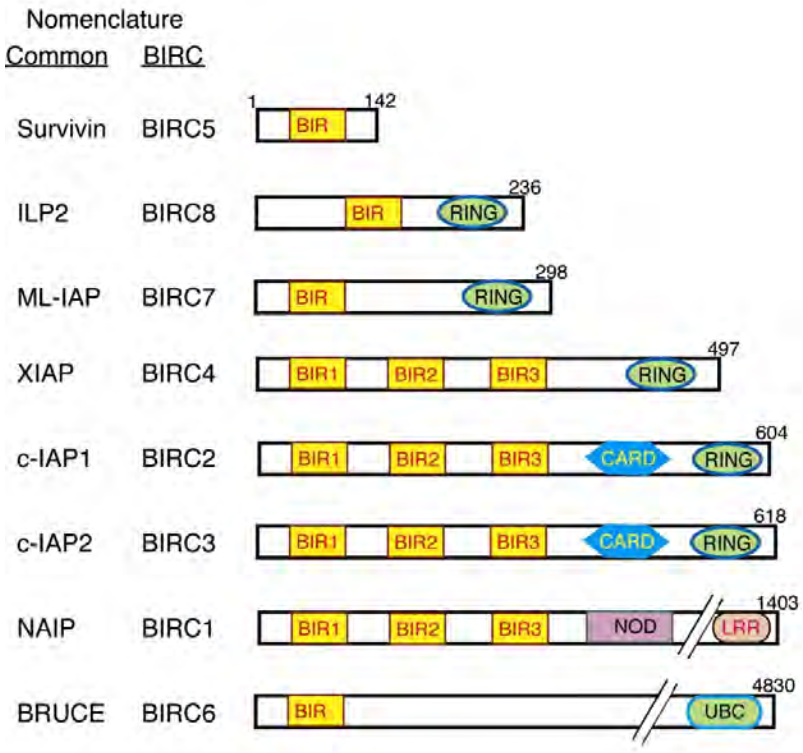

Figure 1-3: Schematic representation of the Human IAP family of proteins

(Srinivasula and Ashwell, 2008)

Survivin, unlike other IAPs, does not bind with caspases directly (Eckelman et al., 2006). Instead, survivin uses alternate pathways such as forming a complex with hepatitis B-X-interacting protein (HBXIP) which inhibits caspase-9 leading to inhibition of mitochondrial apoptosis (Marusawa et al., 2003). Survivin also sequesters Smac, a proapoptotic mitochondrial protein released in the cytosol upon apoptotic induction and hence prevents association of Smac with other IAPs (Song et al., 2003). Also, survivin has been shown to delay the release of Smac/DIABLO from mitochondria when stimulated with DNA damaging agents like etoposide (Ceballos-Cancino et al., 2007).

In addition to these mechanisms, under conditions of apoptotic induction, survivin physically interacts with X-linked inhibitor of apoptosis (XIAP), the only IAP member which directly interacts with caspase 3, 7 and 9. This interaction leads to XIAP stability against ubiquitination and proteasomal degradation (Dohi et al., 2004). This study also showed a synergistic inhibition of apoptosis when stimulated HEK293 cells were transfected with survivin and XIAP together with Bax or Fas, activators of mitochondrial and death receptor pathways respectively. This intermolecular cooperation between IAPs is also seen between survivin and another IAP protein, BRUCE, which regulates cytokinesis (Pohl and Jentsch, 2008).

IAP- IAP interactions are also important in regulating the activity and stability of IAPs. A study by Dohi *et. al*. (Dohi et al., 2007) has shown an important role of phosphorylation status in the regulation of survivin XIAP complex by using survivin mutants that lack (S20A) and mimic (S20E) sites phosphorylated by PKA. Phosphorylation of S20 residue of survivin (Ser20) by cyclic AMP dependent protein

kinase in cytosol (PKA) prevents binding of survivin to XIAP, hence inhibiting the cytoprotective function mediated by survivin and XIAP (Dohi et al., 2007). On the other hand, the mitochondrial survivin which is non-phosphorylatable by PKA binds to XIAP mediating its effects on cytoprotection by inhibition of caspase-9, XIAP stability and enhanced tumor growth (Dohi et al., 2007).

In addition to its role in apoptosis inhibition, survivin plays an important role in mitosis by localizing to various components of the mitotic apparatus like centrosomes, microtubules and midbodies (Fortugno et al., 2002; Li et al., 1998). Physical associations of survivin with polymerized tubulin structures have been shown *in vitro* (Li et al., 1998). Survivin has also been shown to be a part of the chromosome passenger complex formed by Borealin, INCENP and Aurora B kinase (Adams et al., 2001). This complex is involved in proper chromosome segregation (Ruchaud et al., 2007)

Because of its dual role in apoptosis inhibition and mitotic regulation, survivin has emerged as a valuable tool for therapeutic targeting as overexpression of survivin is observed in almost all tumor types tested and has surfaced as the fifth highly expressed transcript in cancer cells in human cancer transcriptome analysis (Velculescu et al., 1999). It has been positively correlated to more aggressive tumors, poor prognosis, high tumor relapse rates and resistance to chemotherapy (Altieri, 2003). Numerous antagonists for targeting survivin are being tested and are summarized in Table 1-3.

Therapeutic approach	Compounds	Preclinical trials	Clinical development
Antisense	LY2181308	Completed	Phase I trial completed Phase II trial ongoing
Molecular antagonists	Ribozyme RNA interference	Ongoing	Not started
Gene therapy	Dominant interfering mutants (C84A; T34A)	Ongoing	Not started
	BIRC5 promoter for tumour-specific transcription of cytotoxic gene(s)‡	Ongoing	Planned
Transcriptional repressors	EM-1421 (tetra-*O*-methyl nordihydroguaiaretic acid)	Completed	Phase I trial ongoing
	YM155	Completed	Phase I trials completed Phase II trials ongoing
Small molecule antagonists of other pathways	STAT3 (STA-21; WP1066)	Completed	Phase I trial planned
	CDK1 (flavopiridol)	Completed	Phase II trial ongoing (NCT00098371)
	TCF (SDX-308)	Completed	Phase II trial ongoing
	HSP90 (17-AAG)	Completed	Phase I and II trials ongoing (NCT00096005, NCT00117988, NCT00096109)
	ERBB2 (lapatinib or Tykerb)	Completed	Phase III trials ongoing (NCT00374322)
Immunotherapy	Autologous CTL pulsed with survivin-primed dendritic cells	Completed	Phase I and II trials ongoing
	Oral DNA vaccine (survivin peptide)	Ongoing	Planned
Peptidomimetic	Combined survivin and HSP90 antagonist (shepherdin)	Ongoing	Ongoing

Table 1-3: Survivin antagonists tested for targeted therapy
(Altieri, 2008)

Structure and Functions of XIAP

XIAP is one of the most extensively studied members of the IAP family. Structurally, XIAP contains three BIR domains, BIR-1, BIR-2 and BIR3 and a RING domain at the carboxy terminus as shown in Figure 1.2. The BIR-1 domain physically interacts with caspase 3 and caspase 7 (Eckelman et al., 2006) and BIR3 binds to caspase 9 (Shiozaki and Shi, 2004). The RING domain, an E3 ligase, mediates the process of ubiquitination leading to the degradation of target proteins including XIAP itself (Yang et al., 2000). Ubiquitination is an important regulatory step as mutants that lack the RING domain (RINGless) are relatively stable and resistant to apoptosis (Yang et al., 2000).

XIAP regulation is also mediated by its interaction with Smac, a mitochondrial protein which is released into the cytosol when a cell undergoes apoptosis. Smac interacts with the BIR3 domain of XIAP, thus competing with the binding site of caspase-9 and releasing the XIAP inhibition of caspase-9 leading to apoptosis. Several peptides that mimic the XIAP binding site on Smac (referred as Smac mimmetics) have been shown to sensitize cells to apoptosis and are being clinically tested (Sun et al., 2008).

Apart from its role as a caspase inhibitor, XIAP also functions as a cofactor in several pro survival pathways. XIAP has been shown to be involved in the activation of TGF-β signaling (Birkey Reffey et al., 2001), stress signaling pathways like N-terminal c-Jun kinase (JNK) pathway and the transcription factor NF-κB (Hofer-Warbinek et al., 2000). XIAP forms a complex with TAB1, a cofactor of TAB associated kinase (TAK1) and leads to JNK activation (Sanna et al., 2002) and NF-κB activation (Lu et al., 2007).

By virtue of its important role in apoptosis inhibition, XIAP has been closely correlated to tumorigenesis. Enhanced expression of XIAP is observed in various tumor cell lines (Yang et al., 2003). In addition, inhibition of XIAP has been demonstrated to sensitize tumor cells to chemotherapeutic agents (Bilim et al., 2003; McManus et al., 2004; Sasaki et al., 2000). Elevated levels of XIAP have also been linked to poor prognosis in several tumors (Mizutani et al., 2007; Tamm et al., 2004). Additionally, XIAP expression has been linked with anoikis resistance in prostate tumor cells (Berezovskaya et al., 2005) and overexpressed in metastatic melanoma (Kluger et al., 2007) pointing to an important role of XIAP in tumor progression. Thus XIAP qualifies itself as an important drug target for anticancer therapy. Several lead compounds and small molecule inhibitors are under clinical trials and are tested for different kind of tumors. The lists of all the XIAP antagonists are summarized in Table 1-4.

Therapeutic compound	Developing organization	Therapeutic Target	Preclinical Stage
Antisense			
AEG35136	Aegera Therapeutics	XIAP mRNA	Phase I Phase II
Peptidomimetic			
Tripeptide	Abbott Laboratories	BIR3	Preclinical
Tripeptide	University of Michigan	BIR3	Preclinical
Natural Product			
Embeline	University of Michigan	BIR3	Preclinical
Synthetic			
Tetrazoyl thioether	University of Texas Southwest medical center	BIR3	Preclinical
C_2–symmetric diyne			
Aryl Sulfonamide	Novartis	BIR2 linker	Preclinical
Polyphenylurea	Burnham institute/TPIMS	BIR2	Preclinical

Table 1-4: Compounds targeted to different domains of XIAP
Modified from (Schimmer et al., 2006)

Section III: Role of NF-κB in tumor progression

Deregulation of several signaling pathways has been implicated in tumor malignancies. Among them, one that is frequently altered in most human cancers is the IκB kinase (IKK)/nuclear factor (NF-κB) pathway (Basseres and Baldwin, 2006). In mammals, NF-κB family consists of five members- RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100) (Ghosh et al., 1998). These proteins exist both as homodimers and heterodimers and their activity is tightly regulated by a set of inhibitory IκB proteins (Hoffmann and Baltimore, 2006). Under normal conditions, NF-κB is sequestered in the cytoplasm as an inactive, IκB-bound complex. Upon stimulation by various agents like TNF-α, interleukins, genotoxic reagents, NF-κB rapidly enters the nucleus and activates gene expression of several target genes (Ghosh and Karin, 2002).

Two pathways of NF-κB activation have been described (Hayden and Ghosh, 2004). The classical pathway, wherein p65-p50 dimers are sequestered in cytosol together with Iκb-α under non-stimulated conditions (Li et al., 1999). On stimulation by various agents, the IκB- α is phosphorylated by IKK complex (serine specific IκB kinase) and hence ubiquitinated and degraded, thereby releasing the p65-p50 complex which then enter the nucleus and regulate gene expression. The alternate pathway involves the upstream kinase NF-κB inducing kinase (NIK) which on stimulation activates IKK complex which phosphorylates and degrades p100, the RelB inhibitor

releasing the RelB-p50 and RelB-p52 complex leading to nuclear translocation of these complexes (Solan et al., 2002).

The key regulatory step for NF-κB activation is the IκB-NF-κB interaction. Tumor associated genetic alterations can disrupt the regulation of NF-κb and IκB proteins in turn causing constitutive activation of NF-κB. Constitutive activation of NF-κb family of transcription factors has been implicated in a wide variety of tumors of breast (Nakshatri et al., 1997; Sovak et al., 1997), pancreas (Wang et al., 1999b), liver (Qiao et al., 2006), prostate (Huang et al., 2001a), and several other cancers. It has been demonstrated to affect all six hallmarks of cancer through transcriptional activation of genes responsible for cell proliferation, suppression of apoptosis, angiogenesis, metastasis and inflammation (Basseres and Baldwin, 2006; Dutta et al., 2006; Jost and Ruland, 2007).

Role of NF-κB in Tumor growth

NF-κB mediates tumor growth and proliferation by the upregulation of several target genes involved in cell proliferation and cell cycle progression. One of the well studied targets of NF-κB mediated tumor cell survival is c-myc (Kim et al., 2000). Transcriptional activation of Pim-2, an oncogenic kinase involved in cell survival requires NF-κB activation (Fox et al., 2003). Similarly, NF-κB mediates the transcriptional activation of cyclin D1, a gene involved in G1 to S transition during cell cycle progression (Hinz et al., 1999). In addition to its role in upregulating genes involved in cell growth and proliferation, NF-κB also regulates the expression of several

antiapoptotic proteins like cIAPs (Wang et al., 2003), Bcl2 family members, survivin and XIAP (Karin and Lin, 2002). The property of NF-κB to inhibit apoptosis is primarily one of the main reasons behind resistance to chemotherapy observed in tumor cells with constitutively active NF-κB (Wang et al., 1999a).

Role of NF-κB in tumor metastasis

NF-κB has also been shown to play an important role in tumor metastasis by activating genes important at different steps of metastatic cascade. It upregulates genes that are important for tumor invasion, like matrix metalloproteinases, urokinase plasminogen activator (uPA) and interleukin-8 (IL-8). MMP-9 expression is transcriptionally regulated by NF-κB as it contains several -κB binding sites (Farina et al., 1999). NF-κB activation also plays an important role in mediating angiogenesis by upregulating the expression of several cytokines (like IL-8) and growth factors (e.g VEGF) (Chilov et al., 1997). Recent studies have shown the involvement of NF-κB activation in mediating EMT (Huber et al., 2004). Repression of E-cadherin expression (Chua et al., 2007) and activation of Twist (Horikawa et al., 2007) by NF-κB strengthens the involvement of NF-κB in mediating EMT. Additionally, NF-κb also activates genes which are important for homing of tumor cells to the distant sites like activation of a chemokine receptor, CXCR4 (Helbig et al., 2003)which is overexpressed in metastatic tumor cells and bind to its ligand, SDF-1 at distant sites leading to metastasis. (Muller et al., 2001)

Section IV: Concluding remarks

It is clear that some of the well studied IAPs particularly survivin is upregulated in most invasive tumors and linked to poor prognosis, reduced survival rates and worse clinical outcome suggesting a strong link to tumor invasion and metastasis (Altieri, 2003). However, the mechanistic link of IAPs to tumor metastasis is still not completely understood and we still lack a clear understanding of whether IAPs contributes to metastasis through their ability to block apoptosis or several additional mechanisms exist that contributes to tumor progression.

In this study, we have focused our efforts in understanding two key questions. Firstly, can the IAPs particularly survivin act as metastasis gene *in vitro* and *in vivo* and what are the essential mechanistic requirements of IAP mediated tumor invasion and metastasis. And secondly, is the metastatic role of IAPs dependent or independent of their known antiapoptotic function (s)? We hypothesized that IAPs play a very different role in metastasis by upregulating a broad gene expression program. This is independent of their anti-apoptotic function and requires a synergistic co-operation of survivin with XIAP, two prominent IAP members, leading to the activation of NFκB in turn activating several genes leading to enhanced metastatic dissemination.

Chapter 2: Characterization of IAP-mediated tumor invasion in vitro

Introduction

Tumor metastasis is the deadliest aspect of cancer as it leads to the majority of all cancer deaths. Several gene alterations are responsible for converting a localized tumor to a more metastatic one. Gene expression profiling is one possible way to identify patients who are likely to develop metastatic cancer during the course of the disease (Fingleton, 2007). Several studies have successfully identified gene targets that contribute positively to this conversion. One such study carried by Todd Golub and his group (Ramaswamy et al., 2003) identified a gene expression signature (128 genes) that discriminated primary and metastatic adenocarcinomas. They showed that solid tumors carrying this signature are more likely to develop metastasis and show worse clinical outcome (Ramaswamy et al., 2003) Among other genes identified in this screen, survivin was identified as one of them. Survivin, a member of IAP family with known functions in apoptosis and mitosis, has been correlated with poor prognosis, reduced survival rates and worse clinical outcome (Altieri, 2003). Survivin has been closely linked to more invasive and aggressive tumors as enhanced expression of survivin is observed in more metastatic phenotypes in hepatocellular carcinomas (Zhu et al., 2005) ,ovarian (Yoshida et al., 2001), colon (Agui et al., 2002) and esophageal (Kato et al., 2001) cancers. Additionally, a study by Grossman *et. al.* (Thomas et al., 2007) showed that transgenic expression of survivin in melanocytes is associated with increased UV induced tumor growth and metastasis *in vivo* suggesting an important role of survivin at both early and

late stages of tumor progression. The mechanistic role of survivin in the process of tumor metastasis is not completely understood although apoptotic inhibition is considered to be one of the mechanisms involved in mediating metastasis (Mehlen and Puisieux, 2006). It is possible that several additional mechanisms are involved in survivin mediated metastasis as a study by Salz *et. al*. (Salz et al., 2005) showed that transgenic mice overexpressing survivin in the bladders is associated with upregulation of several cell adhesion molecules, including fibronectin.

In this part of the study, using various different approaches, we provide evidence that survivin is a 'metastasis gene' which, together with XIAP, plays an important role in tumor migration and invasion via upregulation of gene expression most notably fibronectin. Using cell lines stably expressing IAPs we have dissected the cellular and signaling requirements of IAP mediated tumor cell invasion.

Results

IAP-mediated tumor cell invasion

To study how IAPs may contribute to tumor progression, we stably transfected breast adenocarcinoma MCF-7 cells with a survivin (SVV) cDNA, and selected clones (MCF-7 SVV) expressing 2- to 3-fold increased survivin levels, as compared with parental cultures (Figure 2-1A). Although survivin is an essential mitotic gene, its expression in MCF-7 SVV cells did not affect cell proliferation (Figure 2-1B). Conversely, when added to collagen-coated inserts, MCF-7 SVV cells showed increased cell migration, compared to parental cells (Figure 2-1C). This response could not be attributed to differential adhesion to fibronectin- or collagen-containing substrates

(Figure 2-1D), or changes in actin cytoskeleton (Figure 2-1E), which were indistinguishable in MCF-7 and MCF-7 SVV cells. In addition, and consistent with enhanced cell migration, MCF-7 SVV cells showed dramatically increased invasion across Matrigel-coated inserts, whereas non transfected MCF-7 cells or MCF-7 cells transfected with empty plasmid were not invasive (Figure 2-1F). In order to test if the dramatic invasiveness seen in stably expressing MCF-7 cells is not arising due to clonal selection process, we tested another independent clone of MCF-7-SVV and both the clones showed similar results (Figure 2-1F).

To test whether IAPs participated in this response, we next silenced survivin or XIAP expression in MCF-7 SVV cells by small interfering RNA (siRNA). Survivin- or XIAP-directed siRNA efficiently reduced the expression of the intended target protein, but not vice versa, and a non-targeted siRNA was ineffective (Figure 2-2A). Under these conditions, knockdown of survivin or XIAP nearly abolished MCF-7 SVV cell invasion through Matrigel inserts, as compared with control transfectants (Figure 2-2B). Within the same time frame of the invasion assay, survivin or XIAP knockdown caused only a modest decrease in MCF-7 SVV cell viability (7% and 14.2%, respectively), compared to control transfectants (4.8%) (data not shown). In parallel, we targeted survivin function using a survivin Cys84→Ala/Thr34→Ala double mutant (pAd-T34AC84A). This mutant is unstable *in vivo*, homodimerizes with the endogenous protein and promotes accelerated degradation of the complex (not shown), i.e. it is a dominant negative. MCF-7 SVV cells transduced with adenovirus pAd-GFP or pAd-T34AC84A, expressed comparable levels of GFP, by fluorescence microscopy (Figure 2-2C). In contrast, expression of pAd-

T34AC84A, but not control virus, suppressed MCF-7 SVV cell invasion through Matrigel inserts (Figure 2-2D). Similar to the knockdown results, cells transfected with pAd-T34AC84A showed no changes in cell proliferation or cell survival within the time interval of the invasion assay (Figure 2-2E).

To test whether this response was unique to MCF-7 cells, we then targeted IAPs in different, unrelated tumor cell types. siRNA knockdown of XIAP or survivin in breast adenocarcinoma MDA-MB-231 (Figure 2-3A), or prostate adenocarcinoma cells, PC3 (Figure 2-3C) also abolished their invasion through Matrigel inserts, compared to non-targeted transfectants (Figure 2-3B, D).

To further establish a role of IAPs in tumor cell invasion, we then looked at isogenic tumor cell types carrying homozygous deletion of XIAP. Wild type ($XIAP^{+/+}$) colorectal adenocarcinoma HCT116 cells efficiently invaded through Matrigel inserts, whereas $XIAP^{-/-}$ cells had no invasive potential in this assay (Figure 2-4A). Additionally, we also generated MCF-SVV cell line containing stable knockdown of XIAP. We tested the knockdown efficiency by western blotting (Figure 2-4B). Stable knockdown of XIAP abolished the ability of these cells to invade suggesting an important role of XIAP in tumor invasion (Figure 2-4B).

IAP anti-apoptotic function(s) are not required for tumor cell invasion

To test whether the ability of IAPs to inhibit cell death contributed to tumor cell invasion, we next used a model of rat insulinoma INS-1 cells (Figure 2-5A). Previous studies from our lab showed that stable transfection of survivin in these cells (INS-1 SVV) does not inhibit apoptosis, due to defective mitochondrial import (Dohi et al.,

2007). Regardless, INS-1 SVV cells readily invaded through Matrigel inserts, whereas control INS-1 transfectants were not invasive in this assay (Figure 2-5B). In addition, stable transfection of anti-apoptotic Bcl-2 in INS-1 cells (Figure 2-5A), which promoted exponential tumor growth in vivo, thus confirming its activity (Dohi et al., 2007), did not significantly mediate tumor cell invasion (Figure 2-5B). We next asked whether a similar paradigm applied to XIAP, and we stably transfected $XIAP^{-/-}$ HCT116 cells with a XIAP Asp143Ala/Trp310Ala double mutant (D143A/W310A) that does not bind caspase 3 (D143A mutation), nor caspase 9 (W310A mutation), and is thus devoid of anti-apoptotic functions (Lewis et al., 2004) (Figure 2-5C). Independent clones of this cell line robustly invaded through Matrigel inserts, quantitatively indistinguishably from $XIAP^{-/-}$ cells reconstituted with wild type, i.e. anti-apoptotic, XIAP (Figure 2-5D).

IAP induction of fibronectin regulates tumor cell invasion

To begin understanding how IAPs promote tumor cell invasion, we next looked at the gene expression profile of model MCF-7 SVV cells, in which expression of survivin, alone, was sufficient to confer a highly invasive phenotype (Figure 2-1F). Microarray analysis of these cells revealed a significant upregulation of multiple extracellular matrix proteins implicated in cell invasion, including lumican (↑4106-fold), fibronectin (↑334-fold), and laminin α4 (↑105-fold) (Table 2-1). Accordingly, MCF-7 SVV cells exhibited a >120-fold upregulation of fibronectin mRNA, by real-time PCR (Figure 2-6A), and increased fibronectin promoter activity, by luciferase reporter assay (Figure 2-6B), compared to non-transfected MCF-7 cells. Collagen type 1 α1 and collagen type 5 α2 mRNAs were also increased in MCF-7 SVV cells, albeit less

prominently, whereas expression of laminin 5 was not significantly different, compared to parental cultures (Figure 2-6A). Consistent with these data, MCF-7 SVV cells exhibited a dramatic increase in endogenous fibronectin protein content, by fluorescence microscopy (Figure 2-6C), and Western blotting (Figure 2-6D). In addition, the newly produced fibronectin was readily released pericellularly in the conditioned medium of MCF-7 SVV cells (Figure 2-6E). This was functionally important because MCF-7 SVV cells showed robust migration on Transwell inserts even in the absence of exogenously added substrate (Figure 2-6F), indicating that fibronectin produced and released under these conditions conferred a general migratory phenotype, independent of substrate. In contrast, parental MCF-7 cells did not migrate in the absence of substrate (Figure 2-6F). Similar results were obtained with an unrelated cell type, as INS-1 cells stably transfected with survivin (INS-1 SVV) also exhibited a 7- to 8-fold increased fibronectin mRNA, by PCR amplification (Figure 2-7A), and fibronectin protein, by Western blotting (Figure 2-7B). In contrast, INS-1 Bcl-2 transfectants, or cells transfected with a control plasmid, showed no modulation of fibronectin levels (Figure 2-7 A, B).

Using breast cancer as a model, we next asked whether a relationship between IAP and fibronectin expression existed in human tumors, in vivo. Analysis of two independent published gene profiling studies revealed that survivin and fibronectin were coordinately increased in tumor versus normal tissues (Figure 2-8A, B). This likely reflected expression of fibronectin in the tumor cell population, rather than stromal cells, because additional invasive breast adenocarcinoma cell types, including SUM159 and HBL100 cells, also contained higher levels of endogenous fibronectin (Figure 2-8C).

To begin investigating whether increased production of fibronectin contributed to tumor cell invasion, we employed two approaches, siRNA silencing of fibronectin and antibody blockade experiments. Acute siRNA knockdown of fibronectin in MCF-7 SVV cells (Figure 2-9A) significantly inhibited matrigel invasion by approximately 50% (Figure 2-9B). As a second approach, pre-incubation of MCF-7 SVV with a function blocking antibody to β1 integrin (s) which comprises the main cellular fibronectin receptor on these cells, abolished tumor cells invasion, as compared with non-binding IgG (Figure 2-9 C). These data suggest that β1 integrin recognition of fibronectin, and potentially additional adhesive ligands transcriptionally upregulated in IAP-expressing cells, mediates tumor cell invasion. Conversely, no quantitative changes were observed in overall integrin expression in MCF-7 and MCF-7 SVV cells (Figure 2-10).

Signaling requirements of IAP-mediated tumor cell invasion

To identify downstream signals of IAP-mediated tumor cell invasion, we next focused on kinase cascades implicated in cell motility. Under attached conditions, MCF-7 SVV cells exhibited a constitutively higher level of phosphorylated FAK (Tyr397) (Figure 2-11A), and Src (Tyr416) (Figure 2-11B), compared to parental MCF-7 cells. In contrast, phosphorylated Akt (Figure 2-11C), or ERK1, 2 (Figure 2-11D) was comparable in the two cell types, and total protein content of the various kinases was unchanged (Figure 2-11A-D). We next used the model of transfected INS-1 cells to check the requirement of IAPs in the constitutive activation of these kinases. Compared to control cultures, INS-1 cells expressing wild type survivin exhibited constitutively elevated levels of phosphorylated Src (Figure 2-11E). In contrast, two independent clones of

INS-1 cells transfected with a survivin S20E mutant that does not bind XIAP had no detectable phosphorylated Src (Figure2-11E). This strongly suggests that survivin-XIAP complex is indeed essential for the upregulation of phospho-Src mediated cell signaling.

Functionally, targeting FAK by expressing a dominant negative truncated FAK mutant, FRNK (Figure 2-12A), inhibited MCF-7 SVV cell invasion, compared to control transfectants (Figure 2-12B). Similarly, two pharmacologic inhibitors of Src, PP2 (Figure 2-11C) or SU6656 (Figure 2-12D), abolished Matrigel invasion of MCF-7 SVV whereas pharmacologic antagonists of MEK (PD98059 or U0126), or PI3 kinase (LY294002) had no effect (Figure 2-12C). All the pharmacologic inhibitors tested suppressed phosphorylation of the intended target kinases, validating their specificity (Figure2-12E-G). As control, Src antagonists did not affect cell viability or cell cycle kinetics of MCF-7 SVV cultures (Figure 2-13). The suppression of invasion by PP2 was also seen in another invasive breast cancer line, MDA-MB-231 cells (Figure 2-14).

IAP protection from anoikis

The ability of tumor cells to remain viable when detached from the extracellular matrix, i.e. anoikis, may contribute to metastasis, and a role of the IAP pathway in this response was next investigated. MCF-7 cells maintained in suspension using ultra-low (U-L) attachment plates showed time-dependent anoikis-associated apoptosis (Frisch and Screaton, 2001), as determined by hypodiploid DNA content (Figure 2-15A), and increased released of apoptogenic Smac from mitochondria (Figure 2-15B). In contrast, MCF-7 SVV cells were completely protected from anoikis-induced cell death (Figure 2-15A), and exhibited reduced release of Smac from mitochondria

(Figure 2-15B). When attached to substrate, there was no difference in background levels of Smac release between MCF-7 and MCF-7 SVV cells (Figure 2-15B). siRNA knockdown of survivin in MCF-7 SVV cells in suspension (Figure 2-15C) reversed cytoprotection against anoikis, and strongly enhanced cell death under these conditions (Figure 2-15D). Conversely, survivin silencing in attached MCF-7 SVV cells (Figure 2-15C) did not significantly reduce cell viability within the same time interval (Figure 2-15D).

Although survivin is required to counter anoikis (Figure 2-15D), MCF-7 SVV cells in suspension did not reveal redistribution of survivin from its mitochondrial stores to the cytosol (Figure 2-16A), which is required for apoptosis inhibition (Dohi et al., 2007). Instead, MCF-7 SVV cells in suspension exhibited constitutive phosphorylation of FAK and Src (Figure 2-16B), a mechanism previously implicated in protection from anoikis (Bouchard et al., 2008). In contrast, parental MCF-7 cells did not phosphorylate these kinases in suspension, and total FAK and Src protein content was comparable in both cell types (Figure 2-16B). Confirming the mechanistic requirements of this response, siRNA knockdown of fibronectin (Figure 2-17A), or its receptor, β1 integrin(s) (Figure 2-17B), suppressed FAK and Src phosphorylation in MCF-7 SVV cells maintained suspension. Therefore, similarly to the requirements of tumor cell invasion, IAP suppression of anoikis involves constitutive phosphorylation of FAK and Src via fibronectin-β1 integrin signaling, and this response is independent of tumor cell attachment to substrate.

Discussion

Although well recognized for their role in cytoprotection, IAPs are now viewed as more general regulators of cellular homeostasis (Srinivasula and Ashwell, 2008). In this context, their role in cell motility may be evolutionary conserved, as a *Drosophila* IAP homolog has been implicated in cytoskeletal rearrangement and cell migration (Geisbrecht and Montell, 2004), via activation of small GTPase signaling (Oshima et al., 2006). However, whether a similar model applied to mammalian cells has remained unclear, as recent findings suggested that loss of IAPs, including XIAP, resulted in increased cell motility, potentially via enhanced stability of the c-Raf kinase (Dogan et al., 2008). At variance with these data, we have shown here that IAP signaling is sufficient to convert poorly migratory and non-invasive breast adenocarcinoma MCF-7 cells into a highly mobile and invasive cell type, competent to support extensive metastatic dissemination, *in vivo*. Although it remains possible that this discrepancy may be explained by a different utilization of c-Raf for cell motility (Dogan et al., 2008), rather than cell invasion, which is the process investigated here, it should also be noted that previous studies demonstrated that activated c-Raf (i.e. Raf-1) has a profound inhibitory effect on cell migration (Slack et al., 1999), potentially via inhibition of integrin activation (Hughes et al., 1997), which is required for the pathway of tumor cell invasion described here. Accordingly, evidence obtained here from compounded gene silencing strategies, dominant negative interference, and analysis of IAP knockout cells, *in vitro* and *in vivo*, points to IAP-dependent tumor cell invasion as a general property of disparate cancer types, regardless of genetic makeup or tissue of origin.

One of the pivotal requirements of this pathway was a dramatic upregulation of fibronectin in tumor cells, which involved *de novo* transcription of a proximal, 1.9 kb region of the *fibronectin* gene, and release of newly produced fibronectin protein in the extracellular environment. Overall, this model fits well with the observation that fibronectin is often over-expressed in cancer (Bittner et al., 2000), directly enhancing the metastatic phenotype of tumor cells (Clark et al., 2000), and contributing to the composition of a metastatic niche in the tumor microenvironment (Kaplan et al., 2005). With respect to breast cancer, i.e. a disease model used here, high fibronectin levels have been associated with loss of epithelial polarity (Nelson and Bissell, 2006), increased tissue rigidity (Paszek et al., 2005), resistance to hormonal therapy (Helleman et al., 2008), and disruption of mammary acinar morphogenesis (Williams et al., 2008). There is also evidence that fibronectin-initiated signaling may dictate negative disease outcome, as elevated levels of fibronectin, or its main cellular receptor, β1 integrin(s), have been linked to shortened overall and disease-free survival in breast cancer patients (Yao et al., 2007), as well as high rates of recurrences and distant metastasis in patients with bladder cancer (Dyrskjot et al., 2003). In this context, deregulated IAP expression has been linked to high levels of fibronectin in a transgenic mouse model of bladder cancer (Salz et al., 2005), and in retrospective analysis of breast cancer patients, *in vivo* (this study). In addition, the fact that several, unrelated tumor cell types exhibited constitutively high levels of fibronectin suggests that its expression in microarray analysis of primary tumor specimens reflects a property of the tumor cell population, rather than a differential composition of tumor-associated stroma.

How does fibronectin increase in IAP-expressing cells mediate tumor cell invasion? Data obtained here using function-blocking antibodies, siRNA silencing, and molecular or pharmacologic targeting of signaling kinases suggest a paracrine model, in which fibronectin released from the tumor cells engages β1 integrins at the cell surface, culminating with constitutively high levels of phosphorylated, i.e. activated FAK and Src, required for cell invasion. This is consistent with current paradigms of outside-in signaling by integrins, including β1 integrins (Ginsberg et al., 2005), and their ability to trigger activating phosphorylation of Src (Mitra and Schlaepfer, 2006), and FAK (Sieg et al., 2000), thus conferring invasive potential to disparate cell types, *in vivo* (Parsons et al., 2008). Although it is plausible that additional mechanism(s) (Bialek et al., 2004) may contribute to FAK/Src activation under these conditions, including a potential modulation of growth factor receptor signaling, disruption of a survivin-XIAP complex using a phosphorylation-mimetic survivin S20E mutant (Dohi et al., 2007) strongly reduced Src phosphorylation in IAP-expressing cells. Importantly, this pathway was operational in cells maintained in suspension, thus bypassing the requirement of cell adhesion to extracellular matrix substrates for kinase activation. Under these conditions, IAP-expressing cells forced to remain in suspension were completely protected against anoikis-associated cell death, and this pathway required their expression of survivin. However, suppression of anoikis under these conditions did not result from release of survivin from its mitochondrial, i.e. cytoprotective, stores (Dohi et al., 2007), suggesting that fibronectin-mediated paracrine activation of FAK/Src was sufficient to counter

anoikis (Frisch and Screaton, 2001), and preserve mitochondrial integrity, potentially via PI3 kinase/Akt modulation of Bcl-2 family proteins (Bouchard et al., 2008).

To summarize this part of the study, we have demonstrated that survivin and XIAP both play an important role in tumor invasion in vitro. This is well supported by *in vivo* data described in Chapter 4. Survivin mediated tumor invasion requires activation of a broad gene expression program, leading to activation of various cell adhesion molecules particularly fibronectin. Fibronectin in turn bind to integrin receptors leading to the activation of cell motility kinases, FAK and Src which gets activated leading to enhanced tumor invasion.

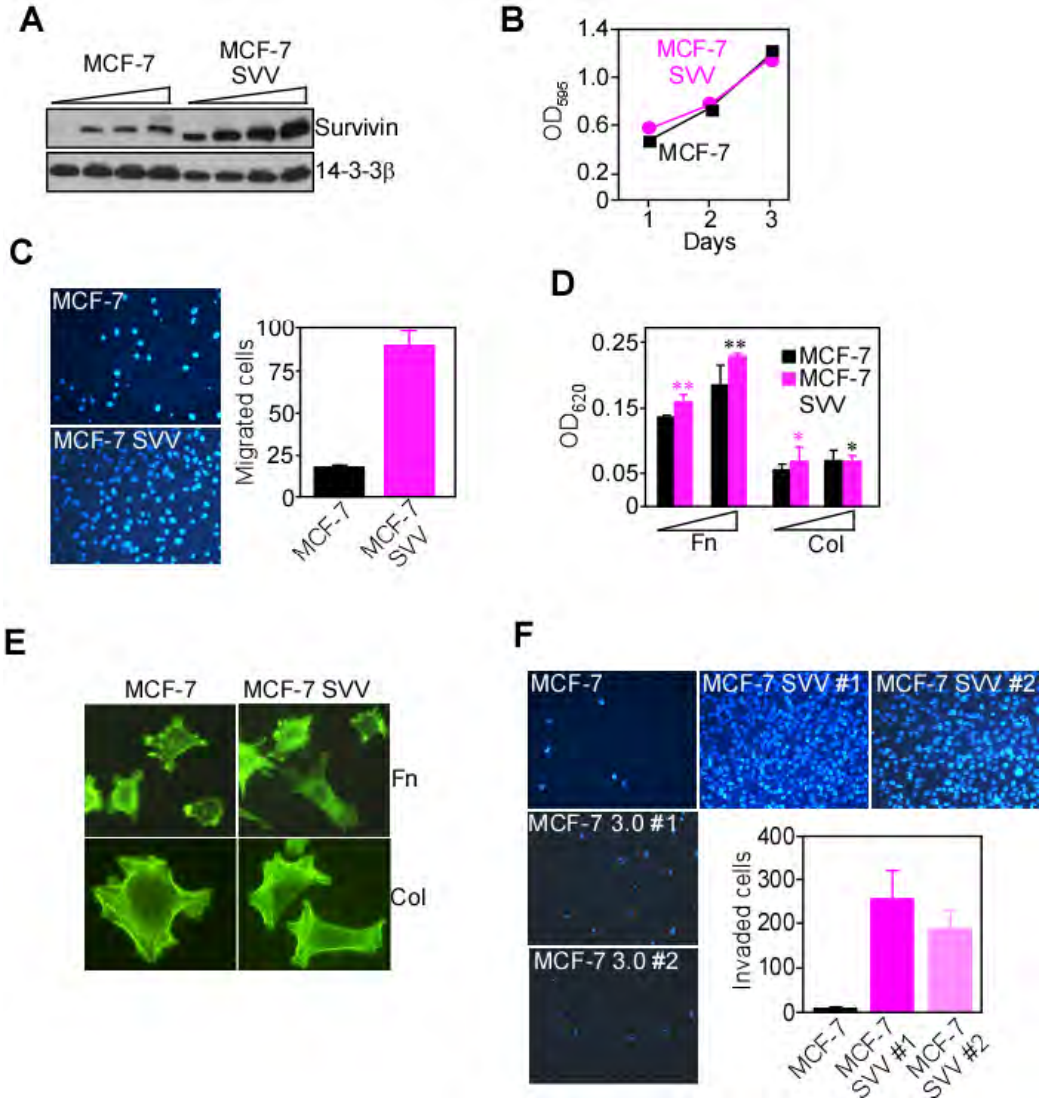

Figure 2-1: Characterization of MCF-7 cells stably expressing survivin

A) Extracts (20-80 ug) from MCF-7 or MCF-7 cells stably transfected with survivin (MCF-7 SVV) were analyzed by Western blotting. (B) MCF-7 or MCF-7 SVV cells were analyzed at the indicated time intervals, by MTT. (C) MCF-7 or MCF-7 SVV cells were added to collagen-coated transwell inserts, and migrated cells were visualized after 1 h by DAPI staining (left), and quantified (right). (D) MCF-7 or MCF-7 SVV cells were added to fibronectin (Fn)- or collagen (Col)-coated plates, and cell adhesion was quantified after 1 h. (E) MCF-7 or MCF-7 SVV cells adherent to fibronectin (Fn) - or collagen (Col)-coated plates were stained with FITC-conjugated phalloidin, and imaged by fluorescence microscopy. (F) MCF-7, two clones of MCF-7 transfected with empty vector or two independently established clones of MCF-7 SVV cells (#1 and #2) were added to Matrigel-coated Transwell inserts, and invaded cells were visualized after 6 h by DAPI staining (left), and quantified (right). For panels C and F, data are the mean±SD of two transwells of a representative experiment out of at least two independent determinations. ** p=0.0081, ** p=0.0091, * p=0.12, *p=0.0062

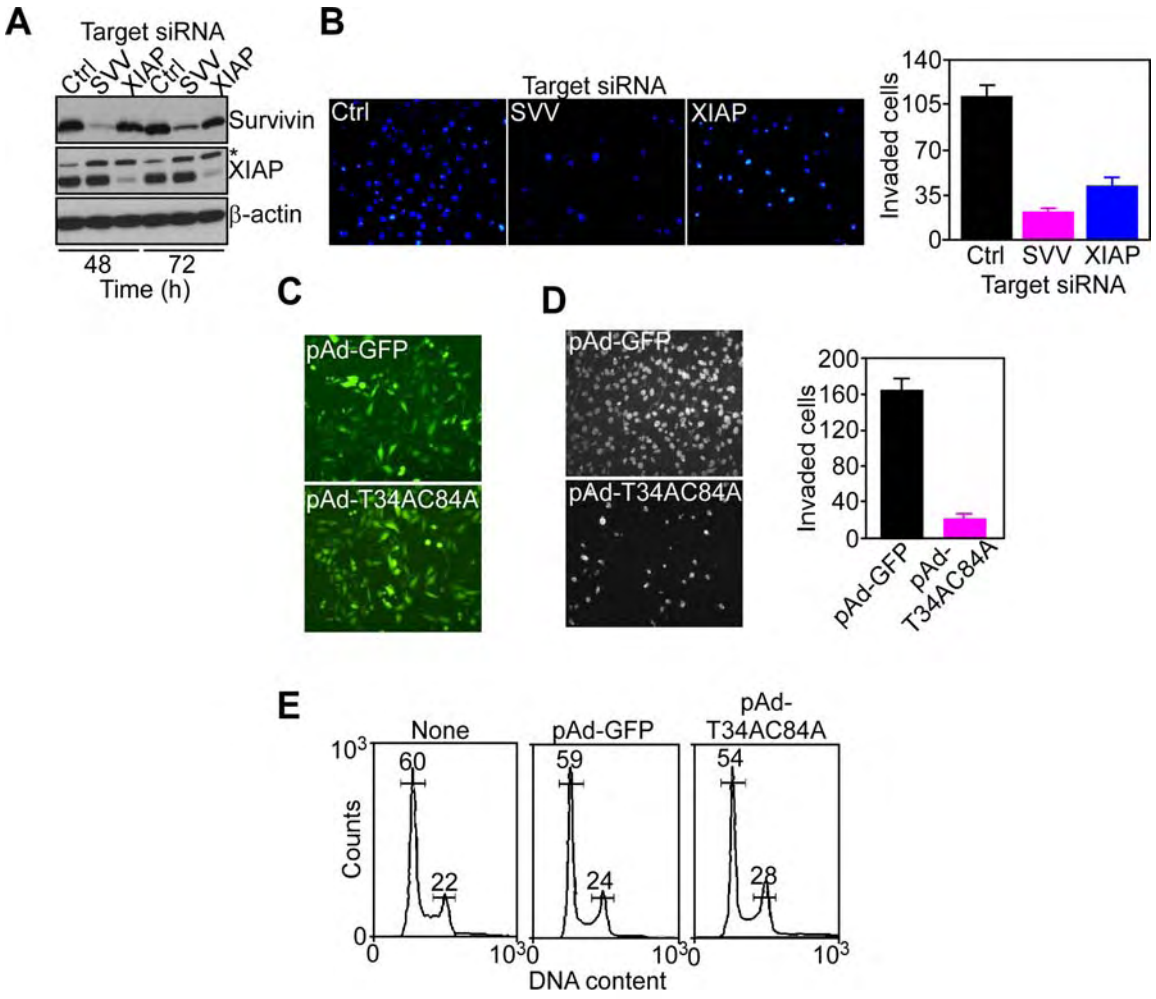

Figure 2-2: IAP-regulation of tumor cell invasion

A) MCF-7 SVV cells were transfected with control (Ctrl) or survivin (SVV) - or XIAP-directed siRNA, and analyzed by Western blotting. (B) siRNA transfected MCF-7 SVV cells that invaded through Matrigel-coated inserts were visualized by DAPI staining after 6 h (left), and quantified (right). (C) MCF-7 SVV cells were transduced with pAd-GFP or survivin pAd-T34AC84A double dominant negative mutant, and imaged for GFP expression after 24 h, by fluorescence microscopy. (D) Transduced MCF-7 SVV cells were analyzed for Matrigel invasion after 6 h by DAPI staining (left), and quantified (right). (E) Untreated or transduced MCF-7 SVV cells were analyzed after 24 h for DNA content by propidium iodide staining and flow cytometry. The percentage of cells in G1 and G2/M transitions is indicated. For panels B and D, data are the mean±SD of duplicates of a representative experiment out of at least two independent determinations.

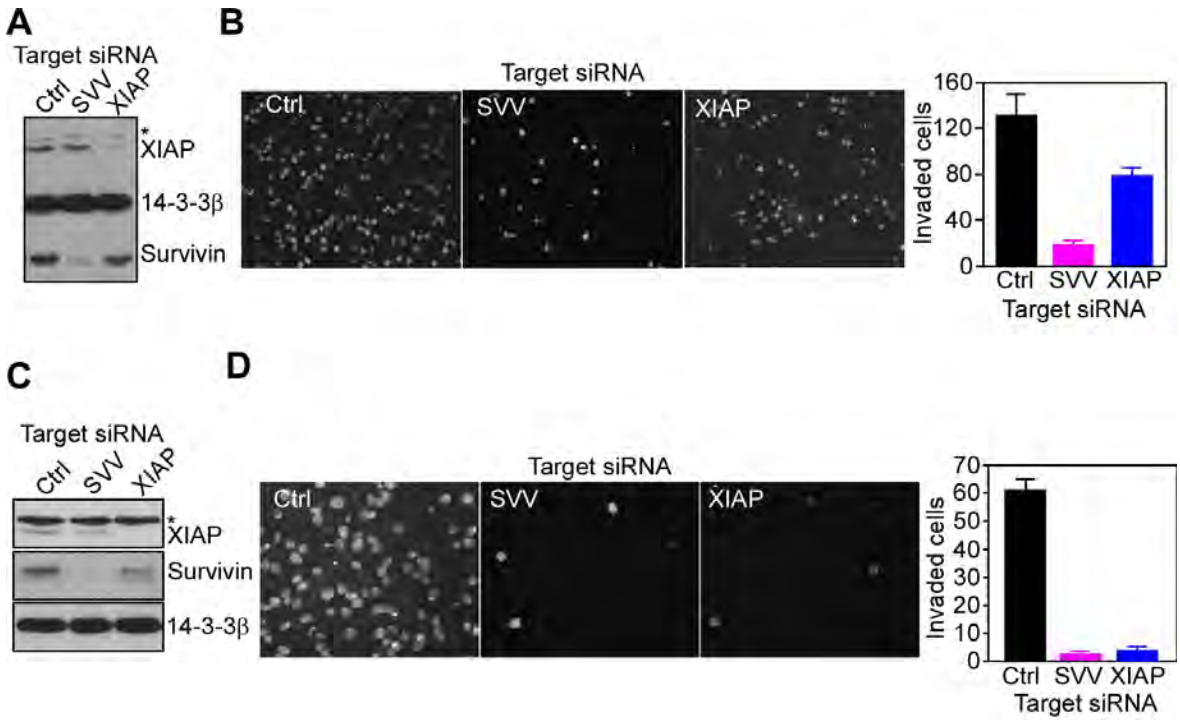

Figure 2-3: IAP-dependent tumor cell invasion

(A, C) Breast adenocarcinoma MDA-MB-231 (A) or prostate adenocarcinoma PC3 (C) cells were transfected with control (Ctrl) or survivin (SVV) - or XIAP-directed siRNA, and analyzed by Western blotting after 48 h. *non-specific. (B, D) Transfected MDA-MB-231 (B) or PC3 (D) cells were analyzed for invasion through Matrigel-coated Transwell inserts by DAPI staining (center), and quantified (right). Data in panels B and D are mean±S.D of two transwells of a representative experiment out of at least two independent determinations.

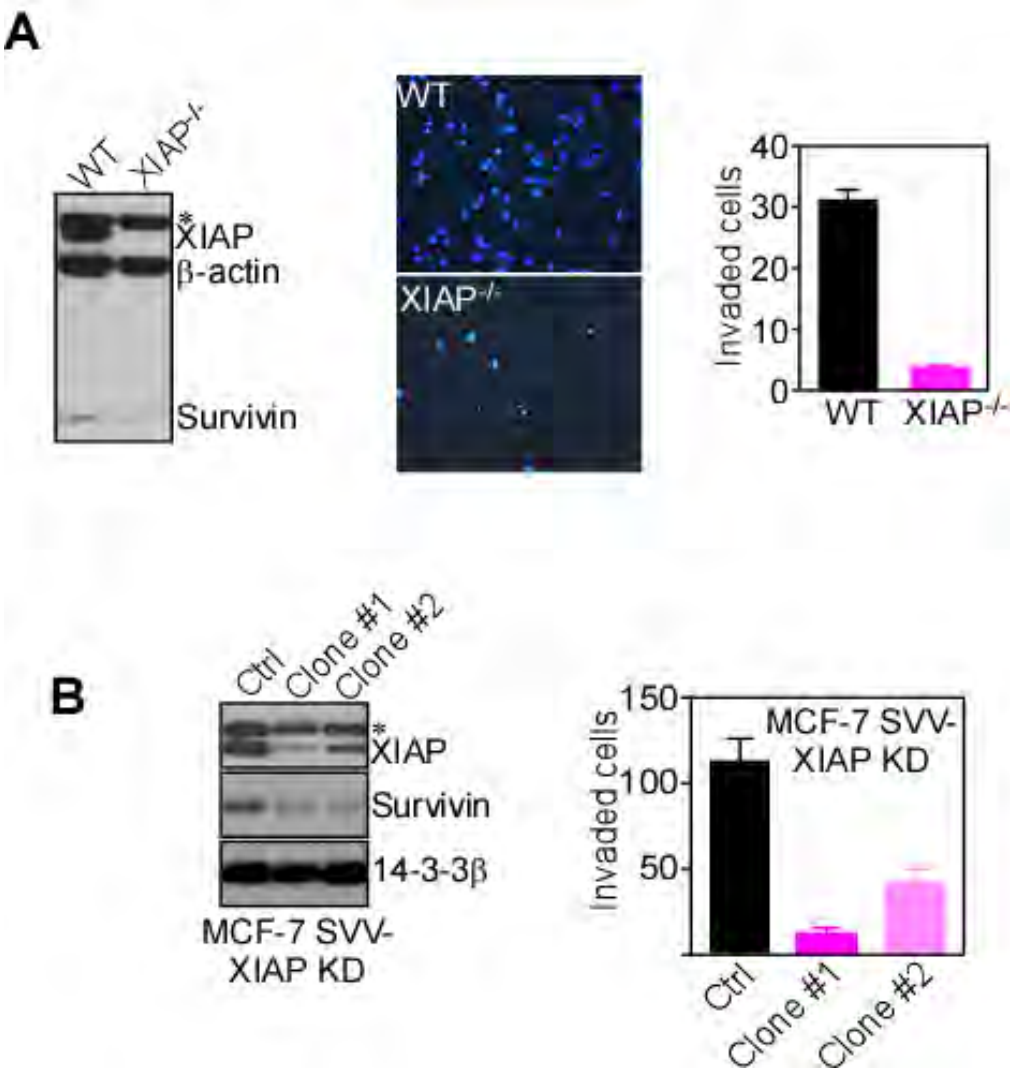

Figure 2-4: XIAP-regulation of tumor cell invasion

(A) Wild type or XIAP-/- HCT116 cells were analyzed for XIAP and survivin levels by immunoblotting. (*left panel*, cells were analysed for matrigel invasion after 6 h by DAPI staining (*middle panel*), and quantified (*right panel*) (B) MCF-7 SVV cells stably tranduced with two independent shRNA clones of XIAP (#1 and # 2) were analyzed by western blotting for XIAP and 14-3-3β (*left panel)* and analyzed for Matrigel invasion after 6 h (*right panel*).

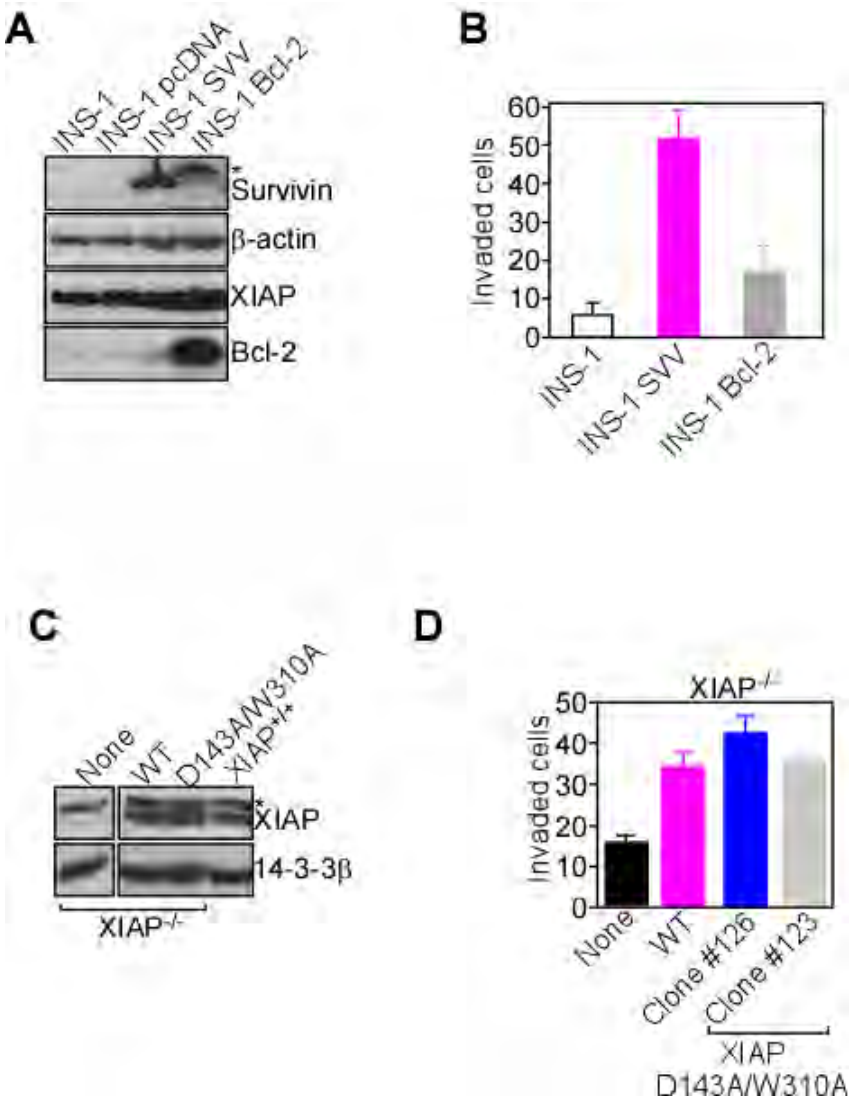

Figure 2-5: IAP mediated tumor invasion independent of its anti-apoptotic function(s).

(A) Rat insulinoma INS-1 cells stably transfected with pcDNA, survivin (SVV) or Bcl2 were analyzed by Western blotting. (B) Stable INS-1 transfectants were quantified for matrigel invasion after 48hrs. (C) $XIAP^{-/-}$ HCT116 cells were stably transfected with WT XIAP or XIAP D143A/W310A mutant, and analyzed by Western blotting. Wild type ($XIAP^{+/+}$) HCT116 cells were used as a control *, non-specific (D) $XIAP^{-/-}$ HCT116 cells stably expressing WT XIAP or XIAP D143A/W310A mutant (clones #123 and #126) were quantified for Matrigel invasion after 6 h by DAPI staining.

		Fold Change	Gene ID	Gene Name
1	Up	4106.7	NM_002345	Lumican
2	Up	334	BC005858	Fibronectin 1
3	Down	302.33	NM_000115	Endothelin receptor type B
4	Up	119	AI123532	Neuronal growth regulator 1
5	Up	105.57	NM_002290	Laminin, alpha 4
6	Up	88.38	AI817041	Chemokine (C-X-C motif) receptor 7
7	Down	85.72	NM_000523	Homeobox D13
8	Down	69.95	AA845258	Biglycan
9	Up	64.58	M21121	Chemokine (C-C motif) ligand 5
10	Up	63.5	NM_016644	Proline rich 16
11	Up	62	BC042995	CKLF-like MARVEL transmembrane domain

Table 2-1: Microarray analysis of MCF-7 and MCF-7 SVV cells

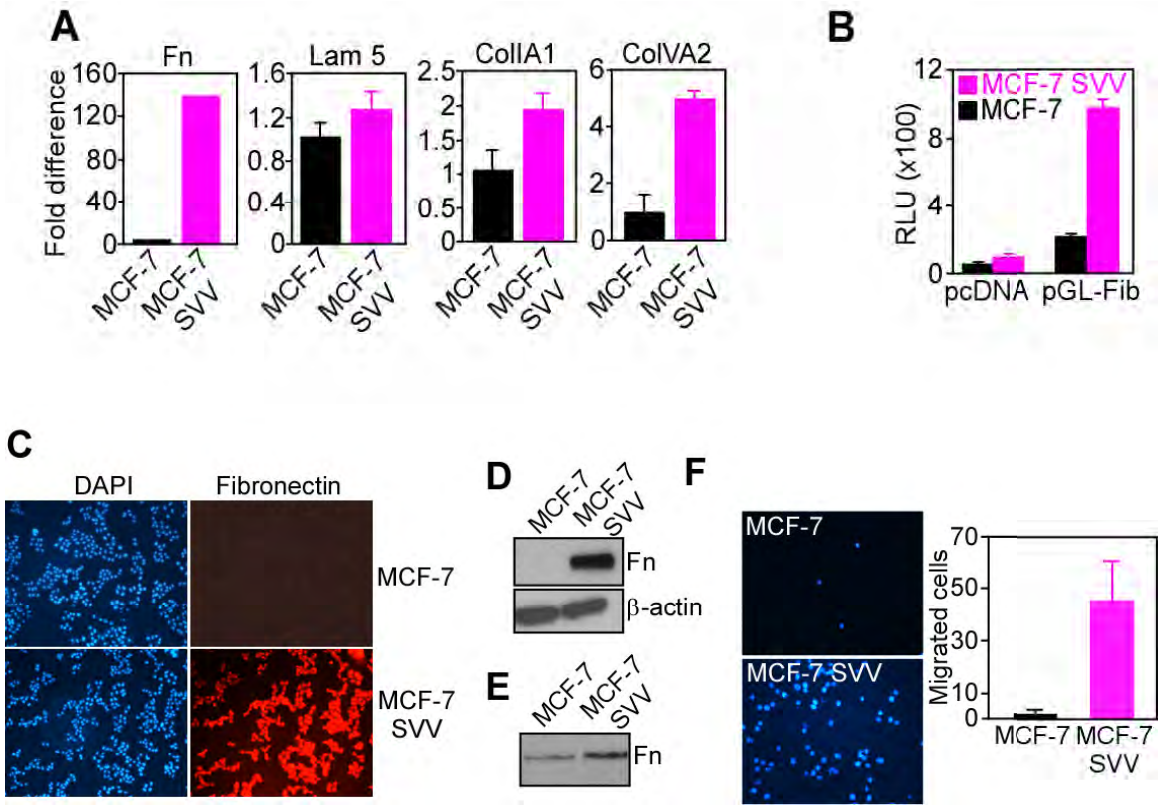

Figure 2-6: IAP mediated induction of fibronectin gene expression

A) RNA from MCF-7 or MCF-7 SVV cells was amplified for the indicated gene products, and normalized to GAPDH expression. Fn, fibronectin; Lam 5, laminin 5; CollA1, collagen A1; Coll VA2, collagen V A2. (B) MCF-7 or MCF-7 SVV cells transfected with pcDNA or a fibronectin luciferase promoter construct (pGL-Fib) were analyzed after 24 h for β-galactosidase-normalized luciferase activity. RLU, relative luciferase units. (C) MCF-7 or MCF-7 SVV cells were stained with an antibody to fibronectin (red), and imaged by fluorescence microscopy, DNA was stained with DAPI (blue). (D) MCF-7 or MCF-7 SVV cells were analyzed by Western blotting. (E) Aliquots of conditioned media isolated from comparable numbers of MCF-7 or MCF-7 SVV cells were analyzed after 48 h by Western blotting. (F) Cell migration on uncoated transwell inserts was analyzed by DAPI staining (*left*), and quantified (right).

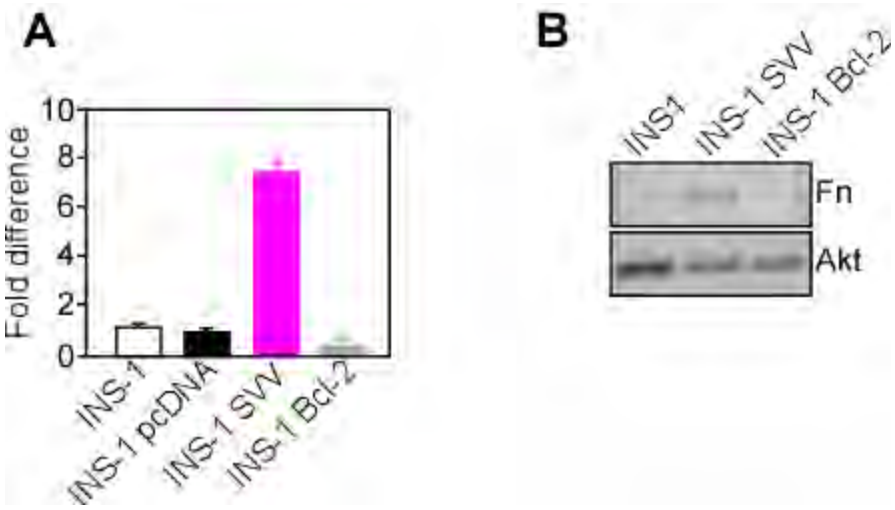

Figure 2-7: IAP regulation of fibronectin in INS-1 cells.

(A) Total RNA extracted from Rat insulinoma INS-1transfectants was amplified with primers for fibronectin, and normalized for GAPDH levels. (B) Parental INS-1 cells or INS-1 stably transfected as indicated were analyzed by Western blotting. Fn, fibronectin.

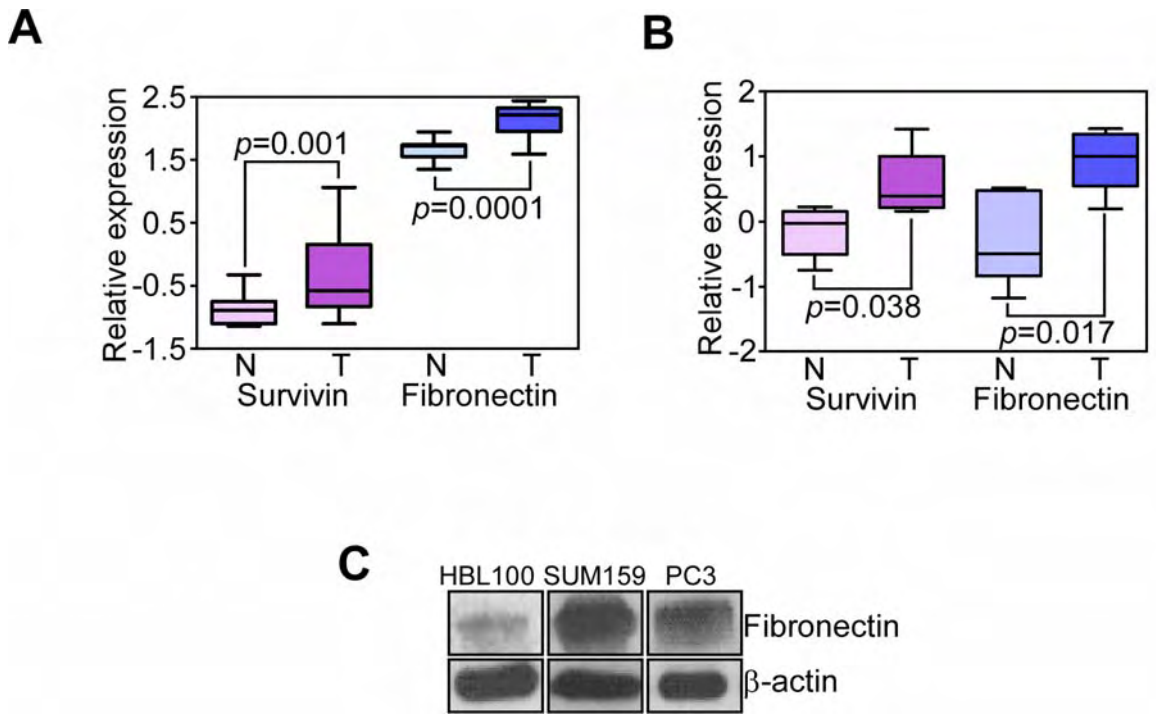

Figure 2-8: Correlative evidence of Tumor-associated fibronectin and survivin expression by microarray analysis

(A, B) Analysis of two published data sets (Richardson et al., 2006) (Turashvili et al., 2007) were analyzed for the expression of survivin and fibronectin in tumor (T) versus normal (N) tissues.
(C) The indicated breast (HBL100, SUM159) or prostate (PC3) tumor cell types were analyzed by Western blotting to see the tumor-associated endogenous fibronectin expression.

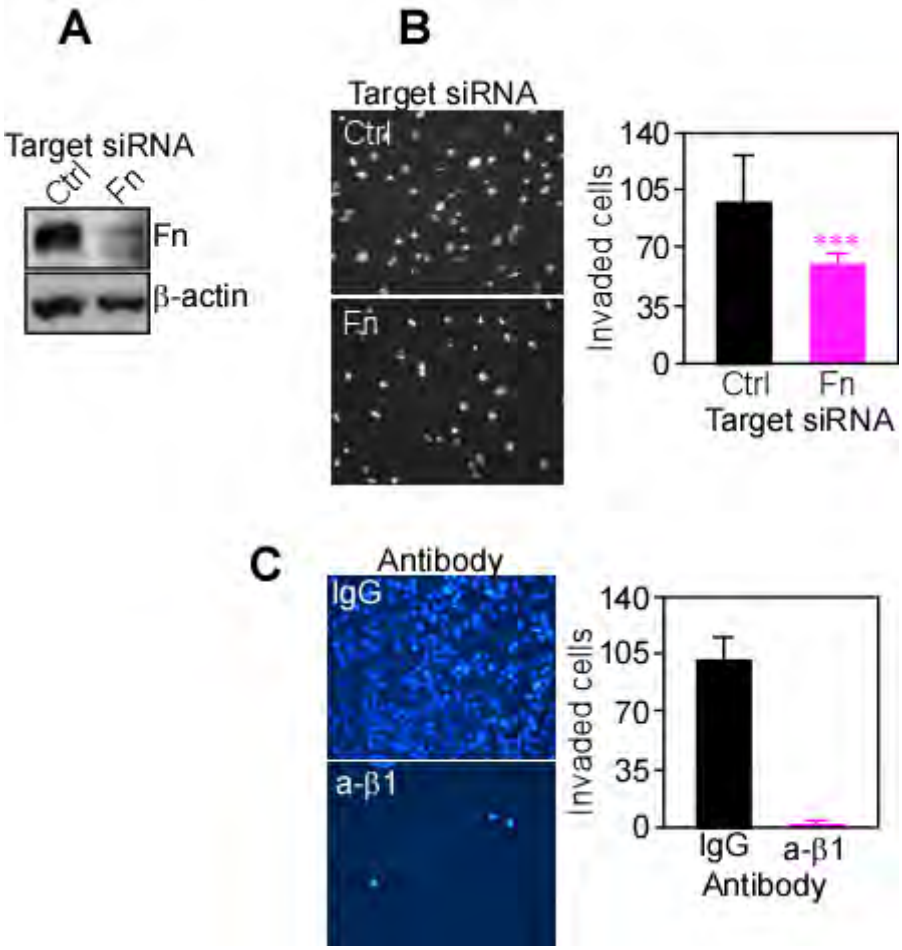

Figure 2-9: Fibronectin regulation of tumor cell invasion

(A) MCF-7 SVV cells were transfected with control (Ctrl) or fibronectin (Fn)-directed siRNA, and analyzed by Western blotting after 48 h. (B) siRNA transfected MCF-7 SVV cells were analyzed for Matrigel invasion after 6 h by DAPI staining (*left*), and quantified (*right*). (C) MCF-7 SVV cells were incubated with a function-blocking antibody to β1 integrin (β1), or IgG, and DAPI stained (left) and quantified for Matrigel invasion after 6 h (right). For panels A and C, data are mean± SD of two transwells of a representative experiment out of at least two independent determinations. ***, p=0.0006

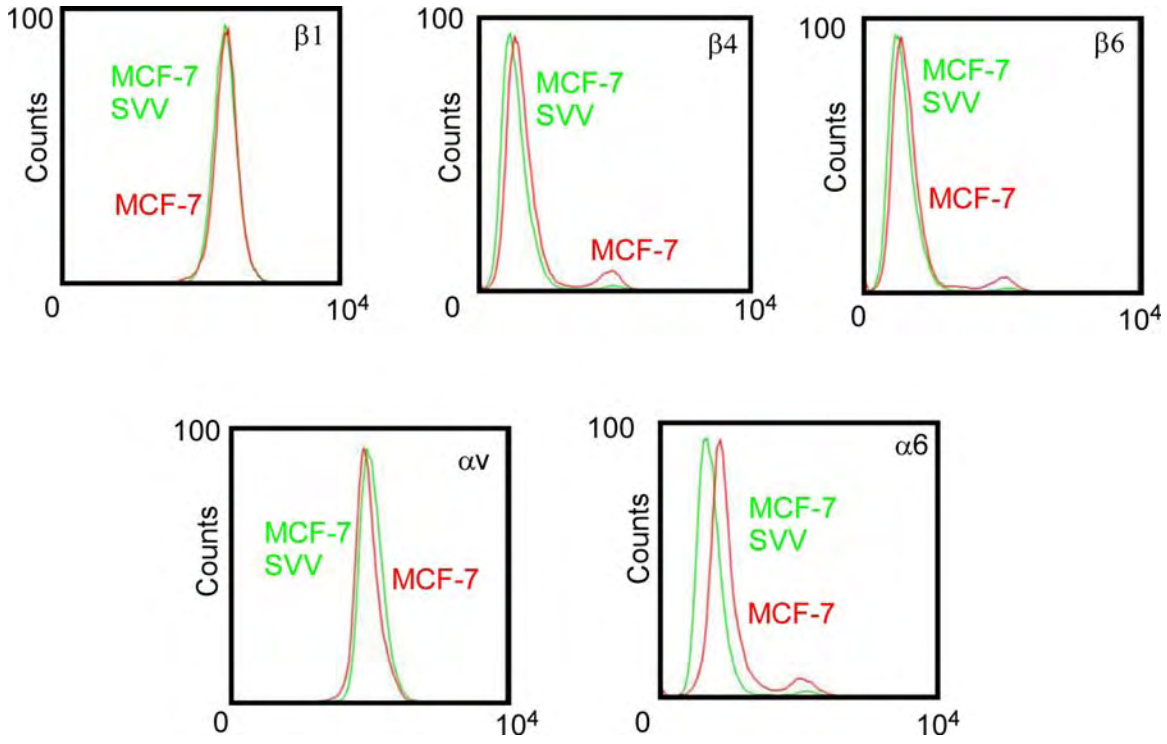

Figure 2-10 : Surface staining of integrins in MCF-7 and MCF-7 SVV

MCF-7 and MCF-7 SVV cells were stained for surface receptors β1, β4, β6, αv and α6 and analyzed by flowcytometry.

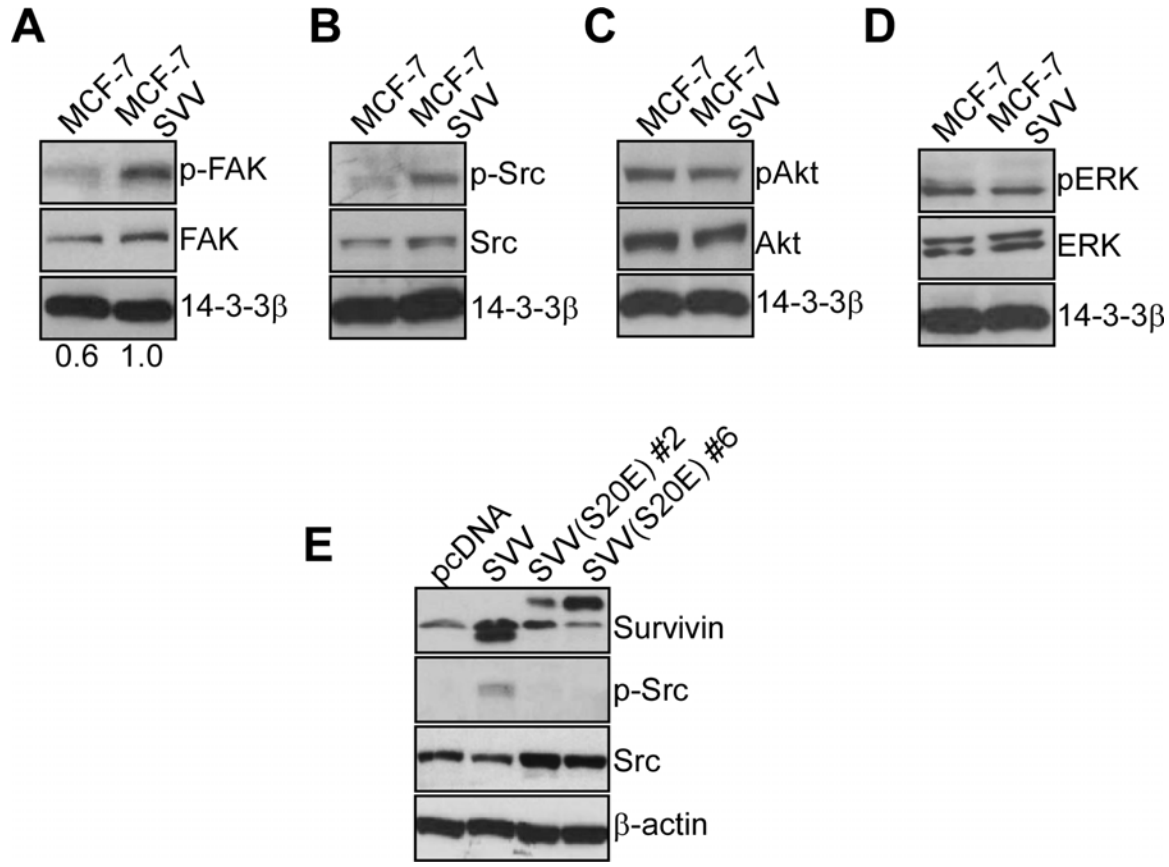

Figure 2-11: Signaling requirements of IAP-dependent tumor cell invasion

A) Adherent MCF-7 SVV cells were analyzed after 48 h by Western blotting for phosphorylation/ expression of FAK (A), Src (B), Akt (C), or ERK1, 2 (D). (E) INS-1 cells stably transfected with wild type survivin (SVV) or two independent clones of survivin S20E mutant (#2 and #6) were analyzed by Western blotting.

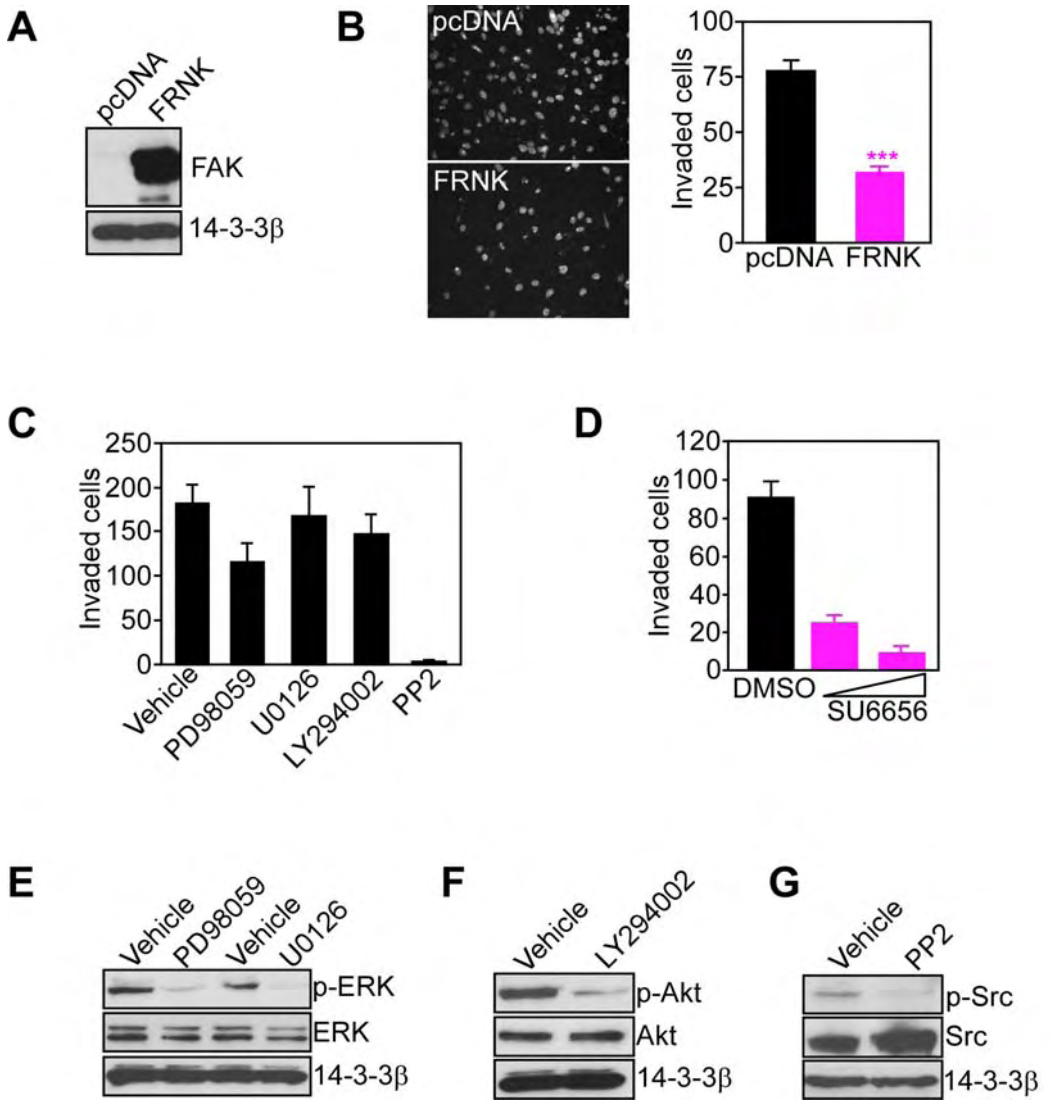

Figure 2-12: FAK/Src-regulation of IAP-mediated tumor cell invasion

MCF-7 SVV cells were transfected with pcDNA or FRNK cDNA, and analyzed after 48 h, by Western blotting. (B) MCF-7 SVV cells transfected with pcDNA or FRNK cDNA were added to Matrigel-coated Transwell inserts, and invaded cells stained with DAPI after 6 h (*left*), were quantified (*right*). ***, $p<0.0001$. (C) MCF-7 SVV cells were treated with the indicated pharmacologic inhibitors, and analyzed in a Matrigel invasion assay after 6 h (D) MCF-7 SVV cells were treated with increasing doses of another Src inhibitor, SU6656 and analyzed in matrigel invasion assay.(E) MCF-7 SVV cells were treated with vehicle (DMSO) or pharmacologic inhibitors of c-Src, PP2 (*left*), PI3 kinase/Akt, LY294002 (*middle*), or MEK, PD98059 or U0126 (*right*), for 24 h, and analyzed by Western blotting

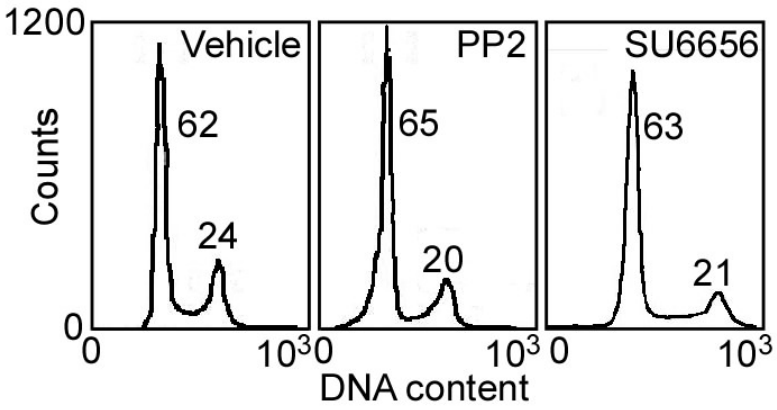

Figure 2-13: Cell cycle analysis after Src inhibition

MCF-7 SVV cells were incubated with vehicle (DMSO) or the indicated Src inhibitors, and analyzed for DNA content after 24 h by propidium iodide staining and flow cytometry. The percentage of cells in the G1 or G2/M phase of the cell cycle is indicated.

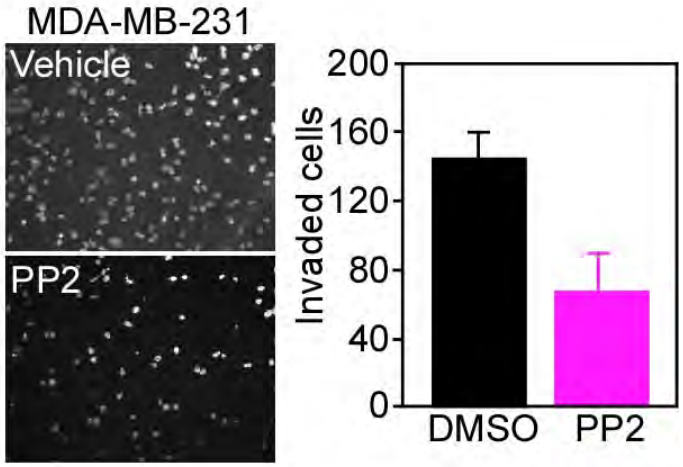

Figure 2-14: Requirements of IAP-dependent tumor cell invasion

MDA-MB-231 cells were incubated with vehicle (DMSO) or Src inhibitor, PP2, and analyzed for invasion through Matrigel-coated Transwell inserts by DAPI staining (left), and quantified (right).

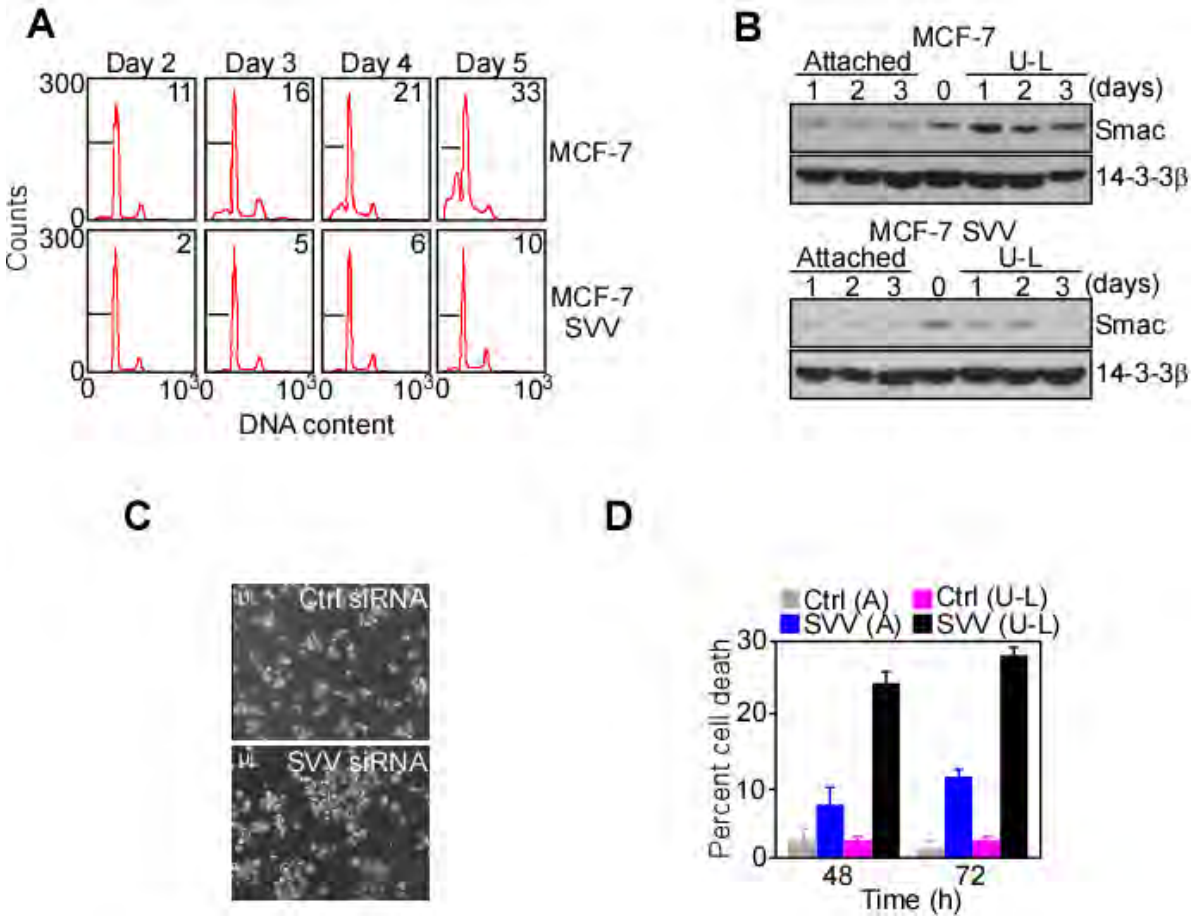

Figure 2-15: Anoikis-associated cell death

A) MCF-7 or MCF-7 SVV cells were forced in suspension using ultra-low attachment plates, and analyzed at the indicated time intervals by propidium iodide staining and flow cytometry. The percentage of cells with hypodiploid (apoptotic) DNA content is indicated. (B) Cytosol extracts of MCF-7 or MCF-7 SVV cells adherent to substrate or in suspension (ultra-low) were analyzed by Western blotting.
(C) MCF-7 or MCF-7 SVV maintained attached or in suspension were transfected with control (Ctrl) or survivin-directed (SVV) siRNA, and analyzed at the indicated time intervals by Western blotting.
(D)Transfected MCF-7 SVV cells were analyzed after 48 h for nuclear morphology of apoptosis under conditions of attachment (A) or in suspension (S). For Panel D, data is mean± SD of at least two independent experiments.

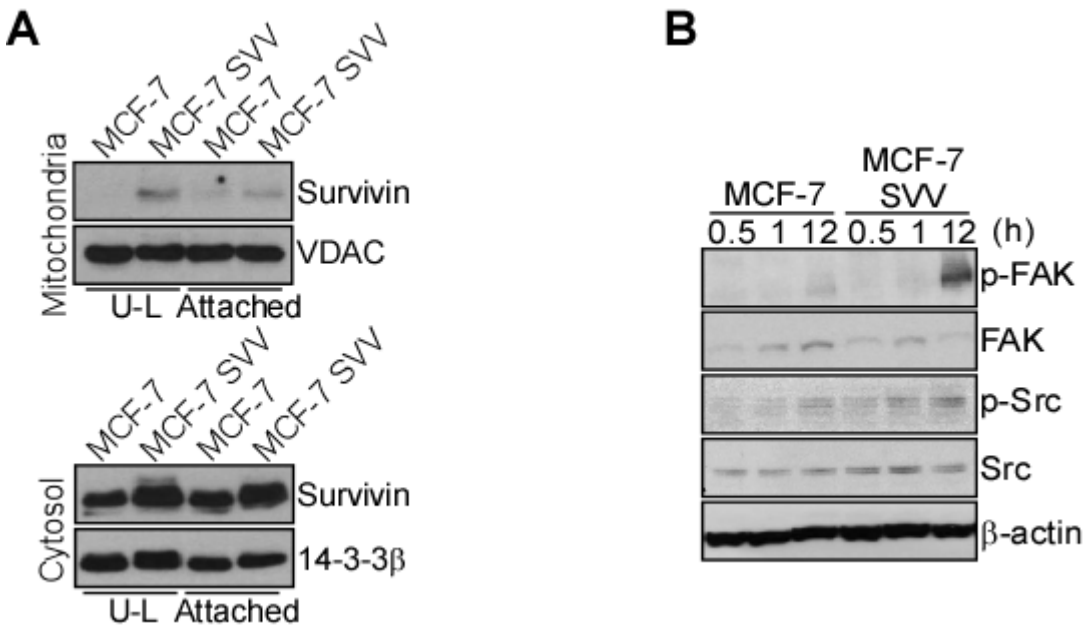

Figure 2-16: Anoikis associated cell signaling

(A) Mitochondria (top) or cytosol (bottom) extracts of MCF-7 or MCF-7 SVV cells attached or in suspension (ultra-low) were analyzed by Western blotting. (B) MCF-7 or MCF-7 SVV cells in suspension were analyzed at the indicated time intervals by Western blotting.

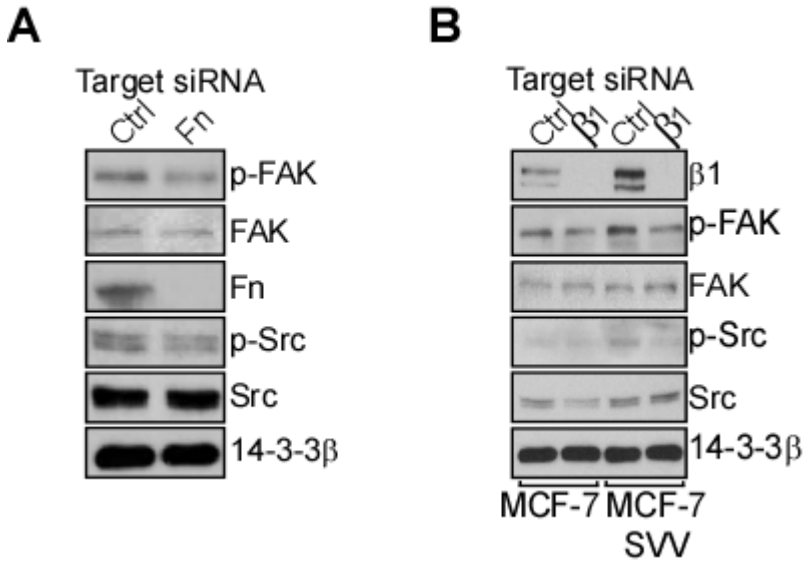

Figure 2-17: Requirement of fibronectin and β-1 integrin in IAP mediated tumor invasion

A) MCF-7 SVV cells transfected with control (Ctrl) or fibronectin (Fn)-directed siRNA in suspension were analyzed by Western blotting. B) MCF-7 and MCF-7 SVV cells were transfected with control (Ctrl) or β-1 integrin siRNA and analyzed by immunoblotting.

Chapter 3: Involvement of survivin-XIAP complex in NFκB activation and enhanced tumor invasion

Statement of contribution

The work presented in this Chapter was done in collaboration with Takehiko Dohi, Research Assistant Professor in our lab. Takehiko Dohi made the stable cell lines expressing different survivin and XIAP mutants which were extensively used for this study. He also performed Luciferase and the electrophoretic mobility shift assays with the stable cells lines as shown in Figure 3-1 and Figure 3.3. The XIAP mutants, RINGΔ, V80D and H467A were obtained from Colin Duckett, University of Michigan.

Introduction

Earlier studies have shown direct interaction of survivin and XIAP under conditions of cellular stress which protects XIAP from polyubiquitination and proteasome mediated degradation *in vitro* and *in vivo* (Dohi et al., 2004). Whether this survivin-XIAP interaction is important in mediating some of the downstream effects of XIAP is not well studied. XIAP is the most well studied IAP member characterized by the presence of three BIR domains which directly interact with caspase 3, 7 and 9 as confirmed by crystal structure (Chai et al., 2001; Huang et al., 2001b; Riedl et al., 2001). XIAP also has a RING finger domain at the carbxy terminus which has E3 ubiquitin ligase activity and is responsible for ubiquitination of other target proteins including itself. In addition to being a caspase inhibitor, XIAP also regulates a number of other

signaling cascades which are caspase independent (Lewis et al., 2004). One of the signaling pathways regulated by XIAP is the NF-κB pathway. The mechanism that has been described recently for NF-κB activation by XIAP is through the formation of TAB1-TAK1 complex which leads to TAK1activation (Lu et al., 2007). TAK1 is MAP kinase kinase kinase (MAP3K) which phosphorylates and activates inhibitor of kB kinase (IKK) (Wang et al., 2001), a kinase responsible for IκB–α phosphorylation and degradation leading to nuclear translocation and activation of NF-κB. XIAP mediated TAK1 activation has been shown to play an important role in mediating anti-apoptotic effects of XIAP (Lewis et al., 2004; Sanna et al., 2002) and cell survival mediated by NF-κB (Hofer-Warbinek et al., 2000). Additionally, TAK1 activation has also been shown to be involved in tumor cell invasion by activation of MMPs (Safina et al., 2008).

Fibronectin (FN), a surface glycoprotein with monomer molecular weight of 220-240 kD is shown to be overexpressed in IAP expressing cells (this study). How the fibronectin expression is transcriptionally regulated in these cells is one of the key questions addressed in this part of the study. Several transcriptional factors and regulatory proteins are involved in controlling FN gene expression in tumor cell types. In melanoma cell lines, fibronectin has been shown to be regulated by Egr-1 transcription factor which gets activated by hepatocyte growth factor (HGF) induced MAP kinase/ERK signaling cascade (Gaggioli et al., 2005). Its expression is also regulated by the protein kinase C (PKC) pathway as seen in hepatoma cells where its expression is increased on stimulation with phorbol 12-myristate 13-acetate (PMA). More importantly several studies have shown that fibronectin serves as a target gene of NF-κB (Lee et al.,

2002; Yi et al., 2000). Fibronectin contains binding sites for p65/p50 homodimer in its promoter region which allows the transcription factor to bind efficiently and regulate the transcription of FN gene (Lee et al., 2002). This is particularly important in the context of this study as we see activated NFκB and enhanced fibronectin expression in IAP expressing cells suggesting a possible correlation between the two proteins.

In this part of the study we have extended the findings of Lu *et. al.* (Lu et al., 2007) demonstrating involvement of survivin-XIAP complex in the activation of NF-κB through the TAB1 TAK1 complex which in turn activate fibronectin expression.

Results

IAP intermolecular cooperation activates NFκB

In this part of the study, we focused on the upstream requirements of IAP induction of fibronectin, as a basis for its subsequent paracrine signaling in tumor cell invasion. For these experiments, we stably transfected survivin in wild type (WT) or $XIAP^{-/-}$ mouse embryonic fibroblasts, which expressed very low levels of endogenous survivin, and selected clones with comparable recombinant protein expression in a $XIAP^{+/+}$ (clones #44 and #68), or $XIAP^{-/-}$ (clones #2 and #5) background (Figure 3-1A). Using this reconstitution strategy, we found that expression of survivin in $XIAP^{+/+}$ cells (clones #44 and #68) stimulated nuclear translocation of p65 NFκB (Figure 3-1B), and a 3-fold induction of NFκB promoter activity, quantitatively similar to TNFα treatment (Figure 3-1C). In contrast, survivin expression in $XIAP^{-/-}$ clones had no effect on NFκB

translocation (Figure 3-1B), or promoter activity (Figure 3-1C). However we observed that a radiolabeled NFκB probe binds to the nuclear extracts of $XIAP^{+/+}$ cells transfected with survivin, by EMSA, and this reaction gets supershifted by an antibody to p65 NFκB (Figure 3-1D). Conversely, expression of survivin in a $XIAP^{-/-}$ background did not result in NFκB-protein complexes, by EMSA (Figure 3-1D). In 'add-back' experiments, transfection of survivin expressing $XIAP^{-/-}$ cells with XIAP restored the formation of NFκB complexes by EMSA, whereas a RING-less XIAP mutant (RING-Δ) was ineffective (Figure 3-1D).

Next, we asked whether IAP stimulation of NFκB contributed to fibronectin expression. Accordingly, siRNA knockdown of p65 NFκB silenced endogenous p65 expression in MCF-7 SVV cells (Figure 3-2A), and suppressed endogenous fibronectin levels (Figure 3-2B). In addition, TNFα stimulation strongly upregulated fibronectin expression in MCF-7 or MCF-7 SVV cells, albeit more prominently in survivin-transfected cultures (Figure 3-2C), consistent with a role of fibronectin as an NFκB target gene. We next carried out two experiments to test a requirement of a survivin-XIAP complex in NFκB induction of fibronectin. First, siRNA knockdown of survivin or XIAP comparably suppressed endogenous fibronectin levels in MCF-7 SVV cells (Figure 3-2D), confirming that both molecules were required for fibronectin induction. Second, INS-1 cells stably transfected with wild type survivin or a survivin S20A mutant that constitutively binds XIAP, exhibited increased NFκB promoter activity in a reaction further enhanced by TNFα (Figure 3-2E). Conversely, clones of INS-1 cells transfected

with survivin S20E, which does not bind XIAP, had significantly reduced NFκB promoter activity with or without TNFα (Figure 3-2E).

Requirement of NFκB activation by IAP intermolecular cooperation

To begin identifying how IAPs activate NFκB, we next focused on potential changes in IκBα, a negative regulator of this pathway. In reconstitution experiments with recombinant proteins, XIAP enhanced phosphorylation of IκBα, *in vitro*, and this reaction was further increased by addition of recombinant survivin (Figure 3-3A). Activation of the MAP 3 kinase, TAK1 has been implicated in this pathway via formation of a complex between XIAP and the adapter protein TAB1 (Lu et al., 2007), and subsequent phosphorylation of the IκBα kinase, IKK. Consistent with this model, XIAP increased IκBα phosphorylation in a reaction enhanced by the proteasome inhibitor, lactacystin (Figure 3.3B). In contrast, a XIAP V80D mutant that does not bind TAB1 (Lu et al., 2007) had no effect on IκBα phosphorylation, with or without lactacystin (Figure 3-3B). Additionally, transfection of WT XIAP in $XIAP^{-/-}$ cells stimulated NFκB promoter activity, whereas a XIAP Val80Asp (V80D) mutant t was ineffective (Figure 3-4A).

To elucidate the structural requirements of this response, we next used XIAP isoforms in reconstitution experiments, *in vitro*. Mixed with extracts of control transfectants, recombinant XIAP increased IκBα phosphorylation, *in vitro* (Figure 3-3C), consistent with the data presented above. In contrast, a truncated XIAP variant lacking the RING domain, or two XIAP point mutants that disrupt the TAB1 binding interface

(V80D), or abolish XIAP E3 ligase activity (H467A) were less effective at mediating IκBα phosphorylation (Figure 3-3C). In parallel, we silenced TAB1 expression by siRNA (Figure 3-4 B), and this completely abolished XIAP-mediated IκBα phosphorylation regardless of the XIAP isoform tested (Figure 3-3 C), and suppressed NFκB promoter activity in the presence or absence of TNFα (Figure 3-3D). siRNA knockdown of the downstream effector of this pathway, TAK1 (Figure 3-4B), also suppressed NFkB promoter activity with or without TNFα (Figure 3-3D). To identify a role of survivin in a TAB1-TAK1 signaling axis, we next carried out co-immunoprecipitation experiments, *in vivo*. Immune complexes of wild type survivin or survivin S20A mutant that constitutively binds XIAP contained co-associated XIAP and TAB1, in vivo (Figure 3-3E). In contrast, immunoprecipitates of survivin S20E mutant, which does not bind XIAP, were devoid of TAB1 (Figure 3-3E), suggesting that a survivin-XIAP complex may optimally recruit TAB1 for subsequent TAK1 activation. Finally, we used two complementary approaches to explore the role of this pathway in tumor cell invasion. In these experiments, siRNA knockdown of p65 NFκB (Figure 3-5A) or expression of a phosphorylation-defective IκBα 'super-repressor' mutant (Figure 3-5B) significantly inhibited MCF-7 SVV cell invasion through Matrigel inserts, compared to control transfectants. As control, expression of the IκBα mutant abolished NFκB promoter activity (Figure3-6), thus validating its specificity.

Discussion

In this study we have shown that upstream requirement(s) for IAP induction of fibronectin depended on stimulation of NFκB activity in tumor cells. This pathway involved de novo transcription of a proximal, 1.9 kb region of the *fibronectin* gene, recapitulated by TNFα stimulation of IAP-expressing cells, and, conversely, ablated by siRNA knockdown of p65 NFκB, thus identifying fibronectin as a bona fide NFκB downstream target gene. Although a role of XIAP in NFκB activation has been recognized previously (Srinivasula and Ashwell, 2008), reconstitution experiments in $XIAP^{-/-}$ cells, siRNA silencing of survivin or XIAP, and analysis of survivin mutants differentially competent or defective to bind XIAP, identified here a novel requirement of survivin in this pathway. Previously, survivin was shown to form a physical complex with XIAP, which resulted in enhanced XIAP stability during apoptosis, and cooperative inhibition of caspase activity (Dohi et al., 2007). The data presented here expand this model, and suggest that in addition to cytoprotection, a survivin-XIAP complex may be also required for IAP signaling, and specifically NFκB-dependent gene expression. The assembly of IAP-IAP complexes may be a more general property of these molecules, potentially useful to add novel functional recognitions, as suggested by the interaction between survivin and IAP protein, BRUCE in the control of cytokinesis (Pohl and Jentsch, 2008).

The mechanisms by which IAPs, typically XIAP, activate NFκB have been debated. Current models for this pathway are potentially operative in a highly context-dependent manner, and involve a ubiquitination step mediated by XIAP E3 ligase activity

(Lewis et al., 2004), parallel activation of Smad4 signaling (Birkey Reffey et al., 2001), or sustained phosphorylation and degradation of the negative NFκB regulator, IκBα (Hofer-Warbinek et al., 2000). The data presented here are broadly consistent with these findings, and suggest that intermolecular cooperation between survivin and XIAP leads to IκBα phosphorylation as a prerequisite for NFκB activation, and subsequent fibronectin-dependent tumor cell invasion. This pathway required the integrity of a TAB1-TAK1 signaling axis (Lu et al., 2007) for efficient IκBα phosphorylation and subsequent stimulation of NFκB promoter activity, *in vivo*. Consistent with earlier observations (Lewis et al., 2004), both the RING domain of XIAP, and its intrinsic RING-associated E3 ligase activity were required for IκBα phosphorylation, potentially via physical docking with the adapter protein TAB1 (Lu et al., 2007), or a ubiquitylation step intercalated in IκBα phosphorylation (Lewis et al., 2004), respectively. As far as a potential role of survivin in this response, co-immunoprecipitation experiments presented here supported a model in which formation of a survivin-XIAP complex may be required to optimally recruit TAB1 in a ternary interaction to facilitate the downstream activation of TAK1, a canonical NFκB activator, (Karin and Greten, 2005), which phosphorylates IKK (Wang et al., 2001), by induced proximity (Lu et al., 2007). Although knockout studies are consistent with this model, and demonstrate that lack of TAK1 results in profoundly impaired activation of NFκB through the canonical pathway, the requirements for TAB1 in this system were less clear, with normal stimulation of NFκB activity observed in $TAB1^{-/-}$ fibroblasts (Shim et al., 2005). In this context, it is possible that a IAP (survivin-XIAP)-TAB1-TAK1 ternary signaling complex may be

preferentially exploited in tumor cells, as opposed to normal tissues, and accordingly, TAK1 has been recently implicated in enhanced migration and metastasis of breast cancer cell types (Safina et al., 2007), thus consistent with the overall findings of these studies.

In this context, there is growing evidence for a role of NFκB signaling in metastasis, largely associated with stimulation of epithelial-mesenchymal transition (EMT), expression of matrix metalloproteinase-9 (MMP-9), and repression of putative metastasis-suppressor genes, *in vivo* (Naugler and Karin, 2008). In our experimental system, IAP-induced NFκB activation did not result in morphological features of EMT, as judged by comparable expression of E-cadherin in MCF-7 or MCF-7 SVV cells, or changes in MMP-2 or -9 levels (Data not shown). Instead, the link between NFκB activation and *fibronectin* gene expression, with its subsequent paracrine activation of FAK/Src signaling and enhanced tumor cell invasion, adds a new mechanistic role of NFκB in tumor progression. From a 'translational' perspective, this suggests that NFκB antagonists (Karin and Greten, 2005) may be beneficial at suppressing fibronectin levels in IAP-expressing tumor cells, thus impairing their invasive potential, *in vivo*. This is consistent with proof-of-concept experiments presented here, showing that siRNA silencing of p65 NFκB or expression of an IκBα 'super-repressor 'mutant efficiently inhibited tumor cell invasion.

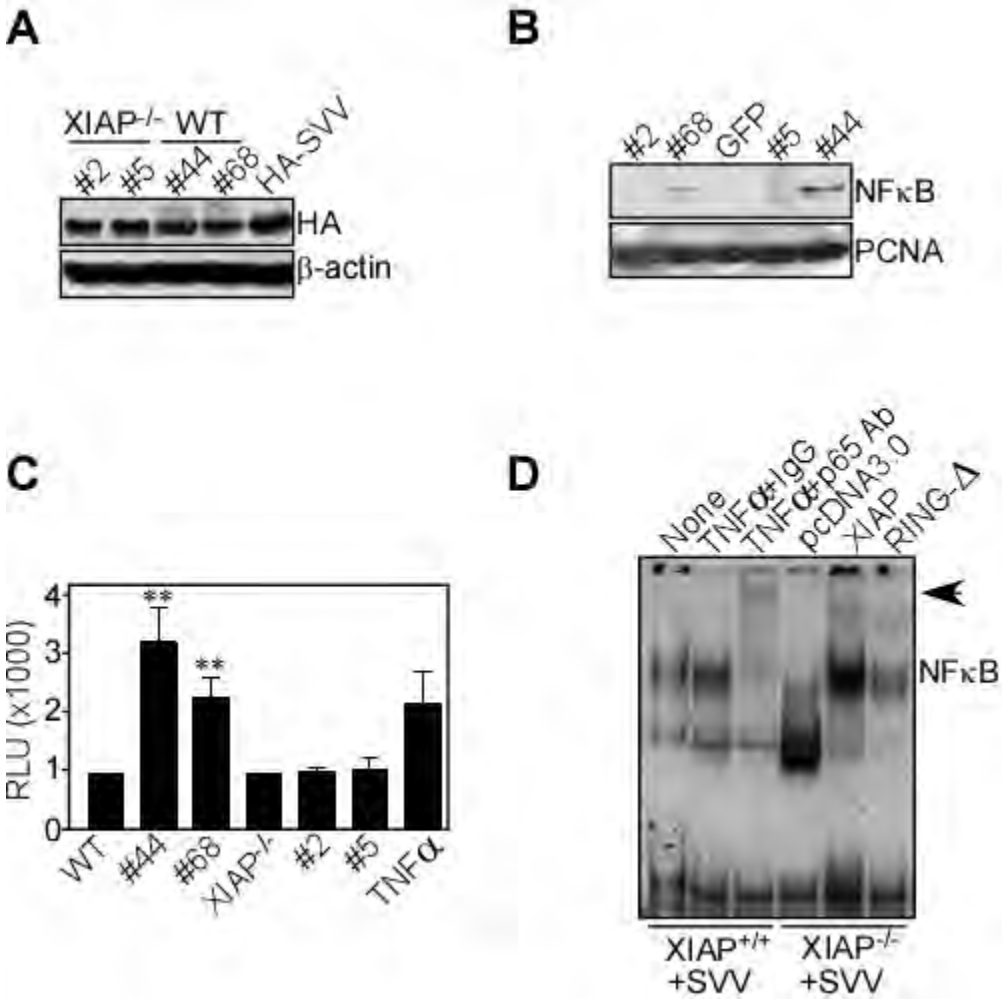

Figure 3-1: IAP activation of NFκB

(A) Wild types (WT) or XIAP-/- MEFs were stably transfected with HA-tagged survivin, and independent clones were analyzed by Western blotting. Cells transfected with HA-survivin cDNA were used as control. (B) Nuclear extracts from MEF clones expressing survivin on a WT (clones #68 and #44) or $XIAP^{-/-}$ (clones #2 and #5) background were analyzed by Western blotting. MEFs stably transfected with GFP were used as control (GFP). (C) WT or $XIAP^{-/-}$ clones expressing survivin were analyzed for β-galactosidase-normalized NFκB luciferase reporter activity. **, p=0.006. (D) Nuclear extracts from WT ($XIAP^{+/+}$) or $XIAP^{-/-}$ clones expressing survivin were incubated with a ^{32}P-labeled NFκB probe in the presence of IgG or an antibody to p65 NFκB, followed by autoradiography. *Arrow*, position of supershifted band. RLU, relative luciferase units. For panel C data are the mean±SEM of replicates out of at least two independent determinations.

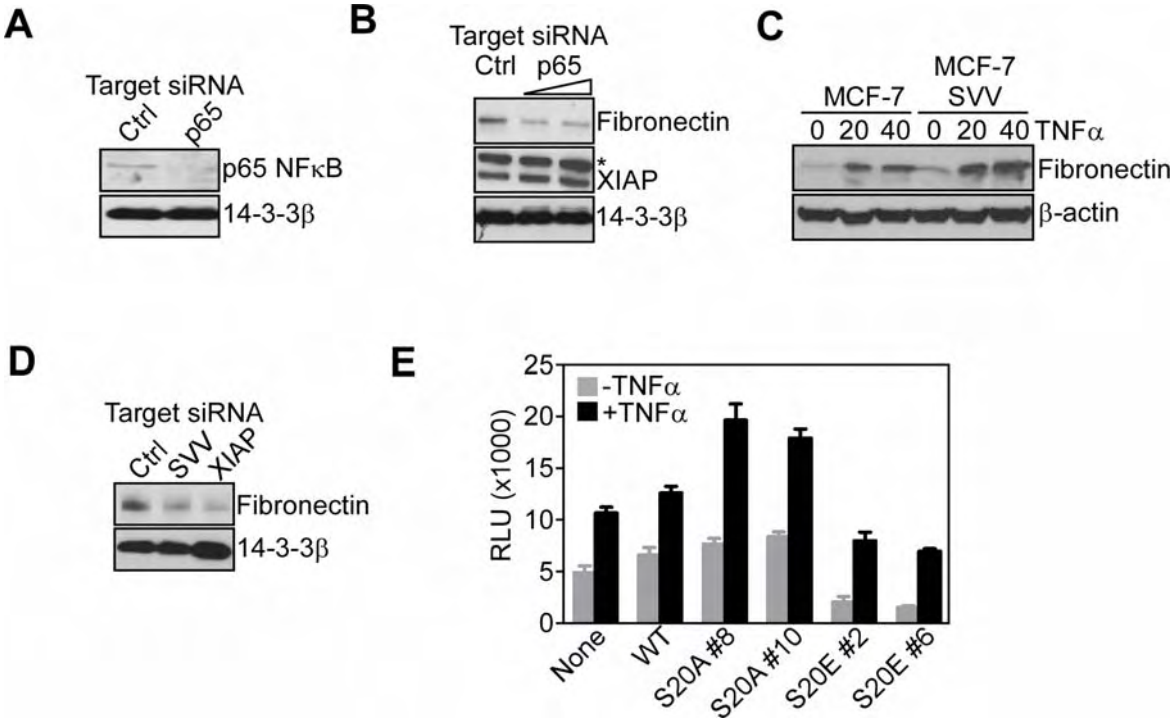

Figure 3-2: IAP mediated NFκB activation of Fibronectin

(A, B) MCF-7 SVV cells were transfected with control (Ctrl), or p65 NFκB-directed siRNA, and analyzed by Western blotting after 48 h (C) Unstimulated or TNFα-stimulated MCF-7 or MCF-7 SVV cells were analyzed by Western blotting ((D) MCF-7 SVV cells transfected with control (Ctrl), survivin (SVV)- or XIAP-directed siRNA were analyzed by Western blotting.(E) INS-1 cells stably transfected with the indicated survivin variants were analyzed for β-galactosidase-normalized NFκB luciferase promoter activity with or without TNFα. RLU, relative luciferase units. For panel E data are the mean±SEM of replicates out of at least two independent determinations

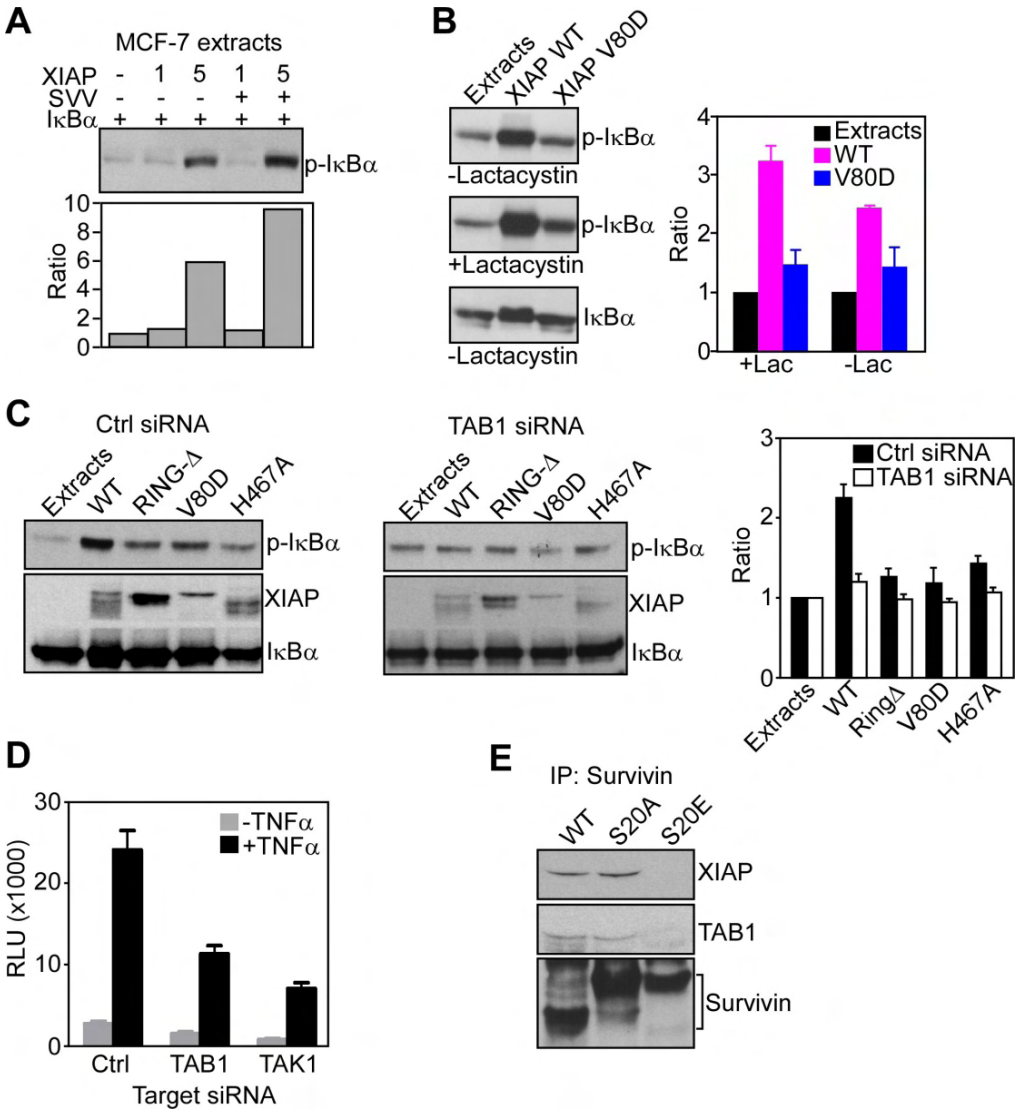

Figure 3-3: Requirements for IAP activation of NFκB.

(A) Extracts from MCF-7 cells incubated with recombinant survivin (SVV), XIAP, or IκBα were analyzed by Western blotting. Bottom, densitometric quantification of protein bands normalized to control lane. (B) The indicated recombinant proteins were mixed *in vitro* with or without the proteasome inhibitor, lactacystin, and analyzed by Western blotting. Right, densitometric quantification of protein bands normalized to control lane. Lac, Lactacystin. (C) Recombinant IκBα was incubated with total cell extracts of XIAP-/- DLD1 cells isolated after transfection with control (Ctrl, left) or TAB-1-directed (center) siRNA, and analyzed by Western blotting. Right, densitometric quantification of protein bands normalized to control lane. (D) MCF-7 SVV cells transfected with the indicated siRNAs were analyzed for β-galactosidase-normalized NFκB luciferase promoter activity with or without TNFα. (E) INS-1 cells stably expressing the indicated survivin variants were immunoprecipitated (IP) with an antibody to survivin and pellets were analyzed by Western blotting.

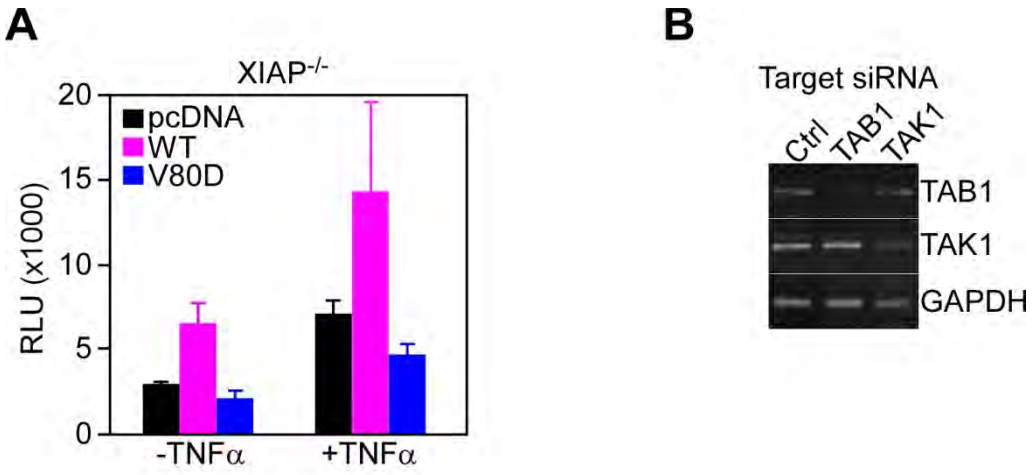

Figure 3-4: Effect of XIAP mutants on NFkB-dependent transcription

(A) XIAP-/- MEF were co-transfected with pcDNA, WT XIAP or a XIAP V80D mutant plus an NFκB luciferase-reporter construct, mixed with or without TNFα, and analyzed for β-galactosidase-normalized luciferase activity. Mean±SEM of triplicates of a representative experiment out of at least two independent determinations. B) MCF-7 SVV cells were transfected with control (Ctrl) or TAB1- or TAK1-directed siRNA, and analyzed by RT-PCR

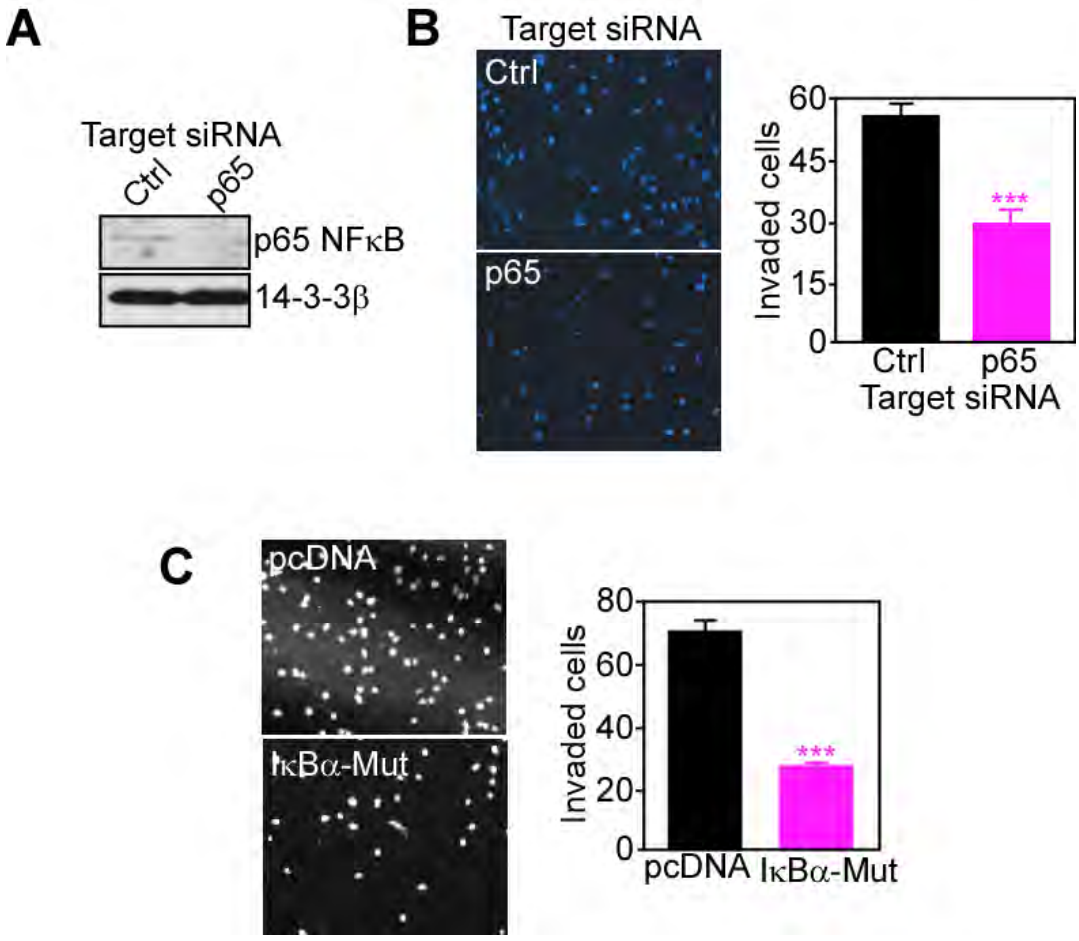

Figure 3-5: Requirements of NFκB for IAP-mediated tumor cell invasion

(A) MCF-7 SVV cells transfected with control (Ctrl) or p65 NFκB-directed siRNA were analyzed by immunoblotting (B) siRNA transfected cells were analyzed for Matrigel invasion after 6 h by DAPI staining (*left panel*), and quantified (*right panel*). (C) MCF-7 SVV cells were transfected with pcDNA or super-repressor IκBα mutant (IκBα-Mut) and analzed for matrigel invasion after 6hr by DAPI staining (*left panel*) and quantified (*right panel*). ***, $p<0.0001$. For panels, B and C, data are the mean ± SD of duplicates of a representative experiment out of at least two independent determinations.

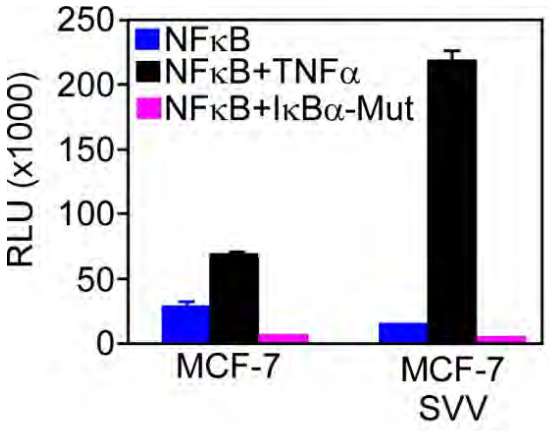

Figure 3-6: Effect of IκBα mutant on NFκB-dependent transcription

MCF-7 SVV cells were transfected with or without a super-repressor IκBα mutant (IκBα-Mut), and analyzed for NFκB-dependent luciferase activity in the presence or absence of TNFα. Mean±SEM of replicates of at least two independent determinations.

Chapter 4: IAP mediated tumor metastasis *in vivo*

Introduction

Metastasis is the most fatal component of all cancers and it is the biggest hurdle to the effective treatment and cure of cancer. Metastasis is associated with poor prognosis and reduced survival rates and is the leading cause of majority of cancer mortalities. It is a complicated, multistep process that requires tumor cells to complete all the necessary steps successfully, including invasion, intravasation, survival in the circulation and finally colonization and growth at distant sites. To understand the process completely we need to use appropriate models that mimic the entire process. *In vitro* approaches are valuable in identifying genes, eliminating variability and enhancing experimental reproducibility but *in vivo* models still remain the 'gold standard' for understanding the process in its entirety. *In vitro* studies simply implicate whether a gene (s) is important in particular step(s) in a metastatic cascade but doesn't necessarily imply that particular gene will help the tumor cells to complete all the steps successfully. Hence appropriate *in vivo* studies are important and required to confirm the *in vitro* findings and understand the complexity of the metastatic cascade.

Several *in vivo* approaches are currently available; the most commonly used among them is the injection of human tumor cell lines in immunocompromised mice including nude mice, SCID mice, and SCID-beige mice in order to prevent immune rejection. For this study we have used SCID-beige mice where the T-cell and B-cell

function is compromised together with NK cell function (Beige mutation). Depending on the site of injection, these transplantable models can be divided into two broad categories.

The spontaneous approach involves injection of tumor cells in heterotopic (or subcutaneous) or orthotopic sites resulting in tumor formation and ‘spontaneous metastasis’. This requires the tumor cells to complete all the steps of metastasis including invasion, escape into the circulation and growth and colonization at distant sites. Thus it mimics the entire metastatic process providing us with a better understanding of the process. For spontaneous assays, orthotopic injection (injecting at the same site from which the tumor was derived) is a preferred method of choice for it enhances the efficiency of metastasis as demonstrated by several studies using breast cancer cells injected in mammary fat pads (Bao et al., 1994; Levy et al., 1982), osteosarcoma injected in bone (Berlin et al., 1993) and prostatic cells injected in prostate (Knox et al., 1993). Orthotopic transplantation also emphasizes the importance of host tumor interactions and tumor microenvironment in tumor progression and metastasis (Joyce and Pollard, 2009).

The second approach involves the injection of tumor cells directly into the systemic circulation bypassing the initial steps of tumor invasion and intravasation. This is referred as ‘experimental metastases’. The common sites used for experimental metastasis is lateral tail vein for studying lung metastasis and intrasplenic or portal vein for liver metastasis. Experimental metastasis assays have several advantages over the ‘spontaneous’ approach. Since the tumor cells are injected directly into the circulation, the metastatic process is rapid and the results are more consistent and reproducible. Secondly, they allow for more focused analysis of the later steps of the metastatic

cascade. Lastly, complications arising due to large primary tumor masses are avoided as sometimes the primary tumor burden overwhelms the animal much before the appearance of macroscopic metastasis. In some cases, primary tumor size strongly correlates with the likelihood of forming metastasis (Price et al., 1990; Safarians et al., 1996). On the other hand, the validity of experimental assays is highly disputed as they fail to provide a true picture of the entire process as they bypass two of the initial steps and key determinants of this process, tumor invasion and intravasation. Secondly, the importance of tumor microenvironment and tumor host interactions are not represented in experimental assays which makes spontaneous assays more ideal, better suited and more efficient way of studying metastasis.

In this study, we chose to use an intrasplenic injection model in which the cells were injected in the spleen and monitored for liver metastasis. This approach is well suited and has been shown to be better than both spontaneous or experimental assays for studying metastasis for various cell lines tested like colon , prostate, melanoma and adenocarcinomas (Kozlowski et al., 1984). It facilitates tumor growth and metastasis in a number of ways that include 1) the absence of a pseudocapsule at the site of injection 2) direct access to the portal vein hence mimicking experimental metastasis to some extent 3) formation of multifocal secondary metastases which characterizes a spontaneous or subcutaneous assay 4) enhanced survival of tumor cells as compared to systemic injection and 5) providing suitable environment for tumor dissemination as observed in certain tumor cells (Kozlowski, 1995)

Results

Intrasplenic injection of breast cancer cells stably expressing survivin

To test whether IAP-dependent tumor cell invasion promotes metastasis, *in vivo*, we used a liver metastasis model in which tumor cells were injected into the spleen of immunocompromised SCID/beige mice; 24hrs later splenectomy was performed to remove the primary tumor mass and mice were monitored for colonization of the liver. Intrasplenic injection of MCF-7 cells in SCID/beige mice did not result in significant liver metastasis, by bioluminescence imaging (Figure4-1A). In contrast, MCF-7 SVV cells injected in the spleen of immunocompromised mice gave rise to extensive metastatic localization of the liver within the same time interval (Figure 4-1A, B). Consistent with this, histologic analysis of livers harvested from mice injected with MCF-7 SVV cells, but not MCF-7 cells, revealed the presence of multiple metastatic nests of epithelial cells (Figure 4-1C).

Intrasplenic injection of HCT116 XIAP-/-

To determine whether a survivin-XIAP complex was required for the metastatic phenotype, we next injected wild type ($XIAP^{+/+}$) or $XIAP^{-/-}$ HCT116 cells in the spleen of SCID/beige mice. Within a 3-week interval, animals injected with $XIAP^{+/+}$ cells exhibited massive metastatic colonization of the liver with nearly complete substitution of the hepatic parenchyma by the tumor cell population (Figure4-2). In contrast, $XIAP^{-/-}$ cells did not significantly metastasize to the liver (Figure 4-2), indicating an absolute requirement of XIAP for this response.

Intrasplenic injection of insulinoma cells overexpressing survivin and Bcl2

Because both survivin and XIAP inhibit apoptosis, we next wished to determine whether the metastatic phenotype of MCF-7 SVV cells was mediated by IAP-dependent tumor cell invasion, as described above, or simply reflected enhanced cell survival. To discriminate between these two possibilities, we injected SCID/beige mice with INS-1 cells stably transfected with survivin or anti-apoptotic Bcl-2, which in previous experiments promoted exponential growth of superficial xenograft tumors, thus confirming its activity (Dohi et al., 2007). Here, animals injected with INS-1 cells or INS-1 stably expressing Bcl-2 did not show changes in blood glucose levels, used as a marker of aberrant insulin production (Figure 4-3A), and produced no or very few liver metastases respectively (Figure 4-3C,D). In contrast, INS-1 cells stably transfected with survivin, INS-SVV caused dramatic and time-dependent decrease in systemic blood glucose level in reconstituted animals, (Figure 4-3A), and this was associated with extensive metastatic dissemination of insulin-producing tumor cells to the liver (Figure 4-3B,C).

Discussion

In this part of the study we provide evidence of the importance of IAP mediated tumor metastasis in vivo. We use intrasplenic injection model for this study as this approach for it allows for an unbiased evaluation of metastatic dissemination, not confounded by the growth of a primary tumor since the spleen containing the primary tumor mass is resected after 24hrs. Using this approach, we demonstrate that expression of survivin and XIAP was sufficient to promote rapid and extensive tumor cell

colonization of the liver, *in vivo*. We have confirmed the metastatic phenotype in two independent cell lines stably expressing survivin, MCF-7 SVV and INS-SVV suggesting that survivin play an important role in the metastatic process of various different kinds of tumors and is not a cell type specific phenomena. Although it remains to be seen whether the higher levels of fibronectin in MCF-7 SVV and INS-SVV cells contribute to this metastatic phenotype, it is clear that both survivin and XIAP are indispensable for this response, given the complete lack of metastatic potential of HCT116 XIAP-/- in this animal model. This is consistent with retrospective analysis of patient series that have consistently associated the expression of survivin and XIAP with aggressive tumor behavior and unfavorable disease outcome, *in vivo* (Hinnis et al., 2007). In addition, IAP-mediated metastasis was independent of the cytoprotective function of these molecules, as expression of anti-apoptotic Bcl-2 could not substitute for survivin in the animal studies, and did not result in systemic tumor growth and appreciable metastatic dissemination to the liver. Previous studies had also suggested that IAP activation of NFκB (Lu et al., 2007) is structurally and functionally separable from its role in caspase inhibition (Lewis et al., 2004), and, accordingly, transgenic expression of survivin resulted in increased rates of metastatic dissemination in a melanoma mouse model, potentially independent of its role in cytoprotection (Thomas et al., 2007).

To conclude, this part of the study confirms the *in vitro* findings of IAP mediated tumor invasion and suggests an important role of these molecules in regulating tumor dissemination by bringing about gene expression changes which help tumor cells to successfully complete the metastatic journey.

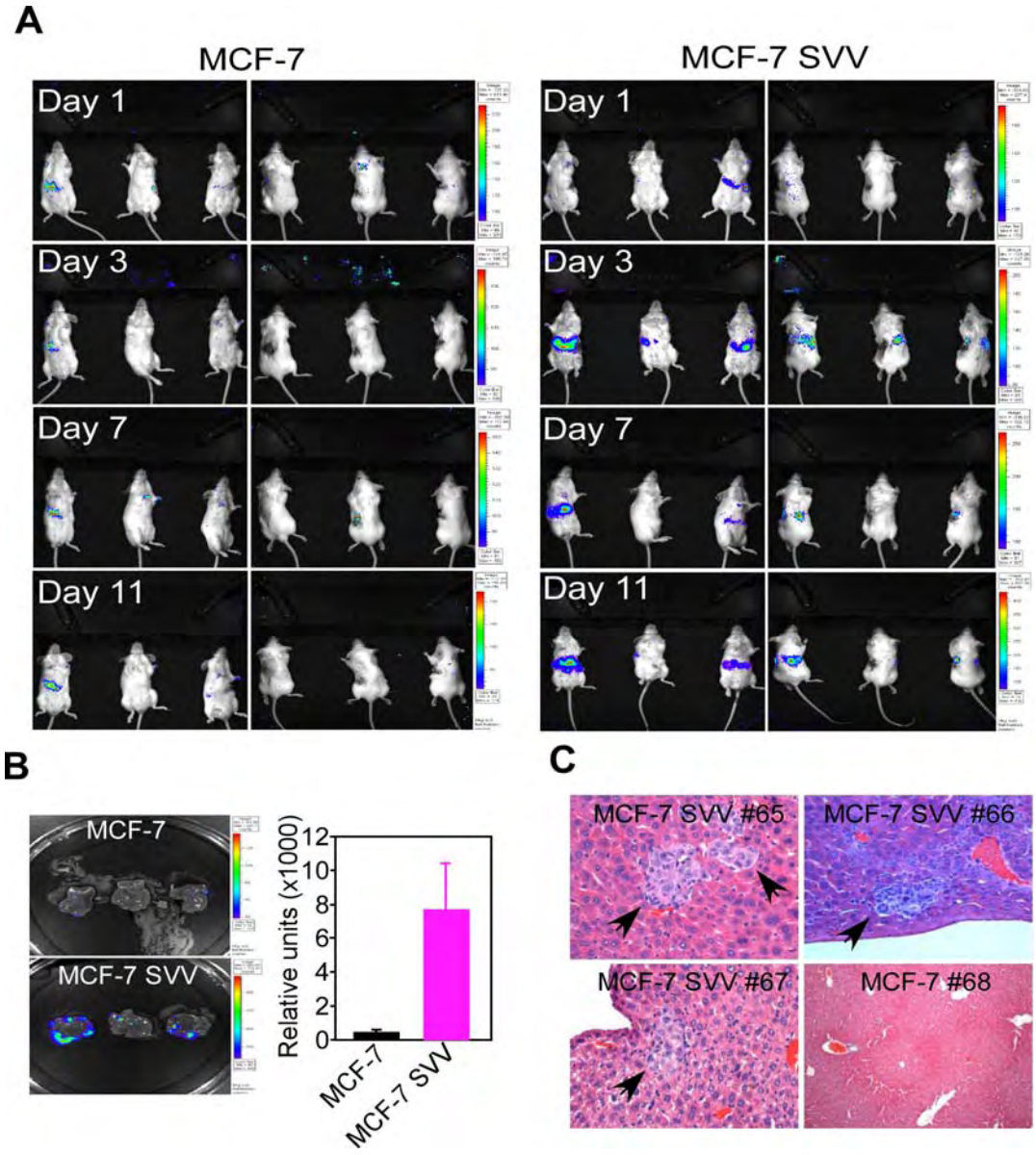

Figure 4-1: IAP-mediated metastasis, *in vivo* using breast cancer cells

A) MCF-7 or MCF-7 SVV cells stably transfected with a luciferase cDNA were intrasplenically injected in SCID/beige mice and analyzed by bioluminescence imaging at the indicated time intervals after injection. B) Livers from mice intrasplenically injected with MCF-7 or MCF-7 SVV cells stably transfected with luciferase were harvested at d. 11 after injection, and analyzed by bioluminescence imaging. *Right*, quantification of fluorescence signals. C) Livers from representative animals (#) injected with MCF-7 or MCF-7 SVV cells transfected with luciferase were harvested at d. 11, formalin-fixed, paraffin-embedded, and analyzed histologically by hematoxylin-eosin staining and light microscopy. *Arrows*, metastatic foci. Magnification, x200,x100.

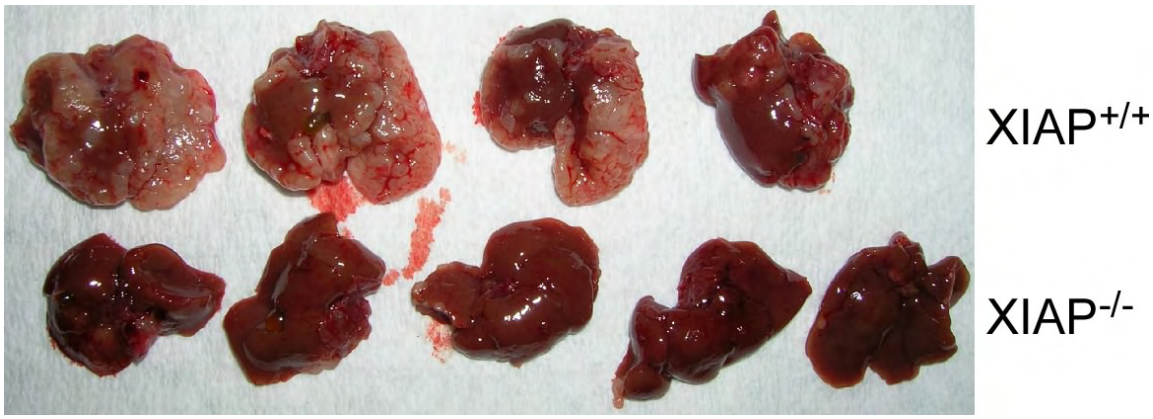

Figure 4-2: XIAP mediated tumor metastasis using colorectal cancer cells

XIAP+/+ or XIAP-/- HCT116 cells were injected in the spleen of SCID/beige mice, and resected livers were examined macroscopically after 3 weeks

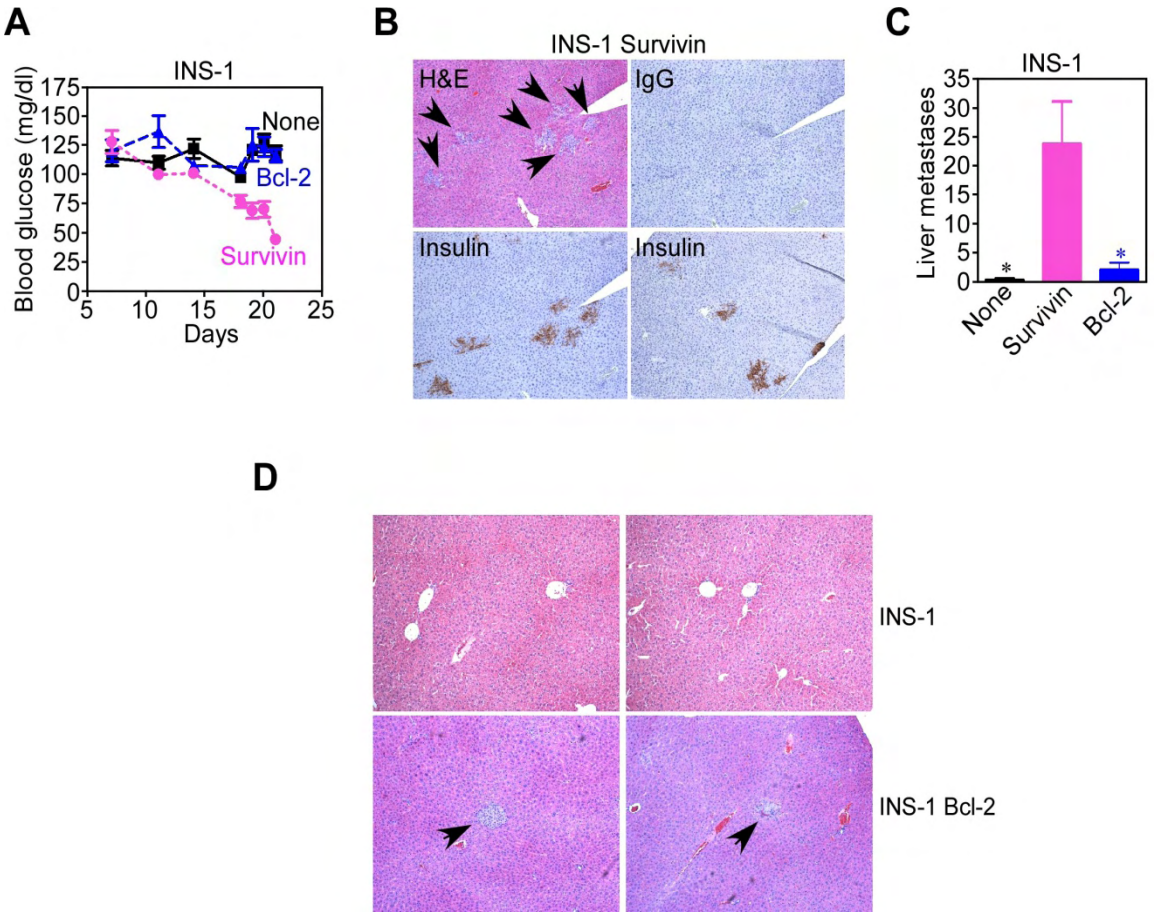

Figure 4-3: IAP-mediated liver metastasis *in vivo* using insulinoma cells

A) SCID/beige mice were injected in the spleen with INS-1 cells expressing survivin or Bcl-2 and monitored for blood glucose levels at the indicated time intervals. B) Livers of animals injected in the spleen with INS-1 survivin transfectants were harvested after 3 weeks, and stained for insulin or H&E. IgG, non binding antibody. Each bar represents metastatic foci/mice. *Arrows*, metastatic foci. Magnification, x100. C) Quantification of liver metastases by INS-1 cells. Metastatic foci were counted blindly in four independent microscopy fields of serial liver sections harvested from the indicated animal groups. *, p=0.017-0.025. D) H&E staining of livers of Wild type INS-1 cells or INS-1 cells stably transfected with anti-apoptotic Bcl-2. *Arrows*, metastatic foci. Magnification, x100.

Chapter 5: Materials and Methods

Cells and cell culture

Wild type or XIAP$^{-/-}$ mouse embryonic fibroblasts (MEF) were the generous gift of Dr. Colin Duckett (University of Michigan, School of Medicine). Colorectal adenocarcinoma HCT116 cells, XIAP$^{-/-}$ HCT116, and XIAP$^{-/-}$ DLD1 were kindly provided by Dr Bert Vogelstein (Johns Hopkins Medical Institutions) and cultured in McCoy's media (GIBCO) with 10% fetal bovine serum (FBS) and 1% pen-strep (GIBCO). Breast adenocarcinoma MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC), and cultured in Dulbecco's modified eagles medium (GIBCO) with 10% fetal bovine serum (FBS) and 1% pen-strep (GIBCO). Prostate adenocarcinomas cells,PC3 and breast adenocarcinomas cells, SUM159 were kindly provided by Dr. Lucia Languino and Dr.Leslie Shaw (University of Massachusetts, Worcester) respectively. They were grown as per ATCC recommendations. The rat insulinoma cell line INS-1 was the kind gift of R.S. Sherwin (Yale University School of Medicine).

Generation of stable cell lines

For generation of MCF-7 SVV stable cell line, breast adenocarcinoma MCF-7 cells were stably transfected with pcDNA3 or a survivin cDNA, transferred to a 100-mm dish in selection medium containing 1 mg/ml G418 (GIBCO), and colonies were isolated 2-3 weeks later. For mice experiments, parental MCF-7 or MCF-7 SVV cells were transfected with pcDNA4/V5-His containing an HA-tagged luciferase cDNA, by Lipofectamine, and stable clones were selected in medium containing 800 μg/ml Zeocin

(Invitrogen) for 3 weeks. Expression of luciferase in stably transfected cells was confirmed by bioluminescence imaging using an IVIS-100 camera system (Xenogen, Alameda, CA). Recombinant protein expression in the various selected clones was confirmed by Western blotting.

For generation of XIAP shRNA stable clones, five Human pLKO.1 lentiviral shRNA plasmids for XIAP (pLKO.1-XIAP) were obtained from OpenBiosystems (cat#; RHS4533-NM_001167). The shRNA expressing lentivirus were cotransfected with 0.5 μg of pLKO.1-XIAP or pLKO.1 (control), 0.33 μg of pCMVdel8.2 and 0.2 μg of pMDG (VSVG envelope glycoprotein) in 293T cells using Lipofectamine 2000 using a 6 well format. Retroviral supernatant were harvested 48hours after transfection, followed by filtration through a 0.45-μm-pore-size filter. Infections were carried out in the presence of 4 μg/ml of polybrene, and cells were selected with 1 μg/ml puromycin for at least one week.

For generation of Ins-1 stable cell lines, INS-1 cells were stably transfected with pcDNA3, wild type survivin, survivin Ser20Ala (S20A) or Ser20Glu (S20E) mutant, or Bcl-2, by Lipofectamine (Invitrogen, 4 μl/well), and clones were selected in medium containing 0.8 mg/ml G418 (Invitrogen), and well characterized in previous studies (Dohi et al., 2007)

RNA quantification

Total RNA was isolated from MCF-7 or MCF-7 SVV cells using RNeasy Mini Kit (Qiagen, Valencia, CA), and cDNA was synthesized using a SuperScript™ First-Strand synthesis system (Invitrogen, Carlsbad, CA), according to the manufacturer's

instructions. Quantitative real time PCR amplification was carried out by TaqMan® detection (Applied Biosystems, Foster City, CA) using the following human and rat primers: fibronectin (Hs01549940_m1), laminin-5 (Hs00245699_m1, collagen type I α1 (Hs00164004_m1), collagen type V α2 (Hs00169768_m1), GAPDH (Hs99999905_m1), rat fibronectin (Rn00569575_m1), rat laminin-5 (Rn01415966_g1), rat collagen type 1 α1(Rn00801649_g1), and rat GAPDH (Rn99999916_s1). Relative gene expression values were calculated by the ΔΔCt method.

Western blotting and antibodies

Cells were harvested and lysed in 20 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 10% v/v glycerol, 50 mM NaF, 1 mM Na_3VO_4, plus protease inhibitors (Roche Applied Science, Indianapolis, IN). Aliquots of cell extracts (30-50 μg) were separated by SDS gel electrophoresis, transferred to nylon membranes, and blocked with 5% dry milk in Tris-buffered saline, 0.1% Tween 20 (TBS-T) for 1 h at 4°C. Samples were incubated with various primary antibodies for 18 h at 4°C in 5% dry milk in TBS-T, washed and further mixed with horseradish peroxidase-conjugated secondary antibodies (Amersham) for 1 h at 22°C. Protein bands were detected by enhanced chemiluminescence (Amersham), and quantified by densitometry.

In some experiments, conditioned medium was collected from MCF-7 or MCF-7 SVV cells ($2x10^5$) after 48 h, concentrated by centrifugation using 100 kD cutoff filters (Millipore Inc, USA), and analyzed by Western blotting. Aliquots of conditioned medium were normalized to cell number prior to loading the gel. The following

antibodies were used: fibronectin (1:1000, H-300, Santa Cruz; and function-blocking 3E3, Chemicon), β1 integrin (1:1000, C-18, BD Transduction Laboratories; and function-blocking P4C10, Chemicon), survivin (1:1000, Novus Biologicals), 14-3-3β (1:5000, Santa Cruz), Bcl-2 (1:1000, Santa Cruz), XIAP (1:1000, BD Transduction), β-actin (1:5000, clone AC-15, A5441, Sigma-Aldrich), focal adhesion kinase (FAK) (1:1000, C-20, Santa Cruz), Tyr397-phosphorylated FAK (p-FAK) (1:1000, Biosource International), c-Src (1:1000, Santa Cruz), Tyr416-phosphorylated Src (p-Src) (1:1000; Biosource International), p44,42 MAP kinase (ERK) (1:1000, Santa Cruz), Thr202/Tyr204-phosphorylated ERK (p-ERK) (1:1000, Cell Signaling), TAB1 (1:1000 Santa Cruz), Akt and Ser473-phosphorylated Akt (1:1000, Cell Signaling), p65 subunit of NFκB (1:1000, Santa Cruz), IκBα (1:1000, Santa Cruz), and p-IκBα(1:1000, Cell Signaling), Smac (Novus Biologicals, 1:1000), and cytochrome c (Clonetech, 1:100).

Transient transfections

For gene silencing by small interfering RNA (siRNA), various cell types were transfected with control non-targeted, or double-stranded RNA oligonucleotides (100 nM/well, Dharmacon) directed to p65 NFκB (SMART pool L-003533-00-0010), fibronectin (SMART pool L-009853-00), XIAP (SMART pool M-004098-01-0020), TAB1 (SMART pool M-004770-02-0005),TAK1 (SMART pool M-003790-06-0005) or survivin using Oligofectamine (3 μl/well), as described (Dohi et al., 2007). For DNA transfections, 4 μg of plasmid DNA was transfected in the various cell types using

Lipofectamine 2000 (Invitrogen, 6 μl/well). After 24 h at 37°C, cells were washed, and analyzed for protein expression by Western blotting or RT-PCR.

Immunofluorescence

MCF-7 or MCF-7 SVV cells grown on optical grade glass coverslips in fibronectin-depleted medium were fixed in 4% paraformaldehyde for 30 min at 4°C, and permeabilized with 0.5% Triton X-100 in PBS, pH 7.4, for 30 min at 22°C. After blocking with 3% BSA plus 0.2% Tween 20 in PBS, pH 7.4, samples were incubated with an antibody to fibronectin for 1 h at 22°C, mixed with an FITC-conjugated secondary antibody, and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). Slides were analyzed under a fluorescence microscope (Axioplan 2, Zeiss) equipped with a charge-coupled device camera (Axiocam, Zeiss). In some experiments, MCF-7 or MCF-7 SVV cells were fixed in 4% paraformaldehyde, stained with Texas red-phalloidin (1:200, Molecular probes), and analyzed by fluorescence microscopy. Images were analyzed using Photoshop CS2.

Immunoprecipitation

For immunoprecipitation, cells were lysed in immunoprecipitation buffer (IP buffer) containing 50 mM Tris, pH 7.5, 50 mM NaCl, 1% Nonidet P-40, 0.1% CHAPS, plus protease inhibitors (Roche Applied Science), and 1 mM Na_3Vo_4 and 50mM NaF. The cell lysates were incubated with IgG or an antibody to TAB1 or TAK1 and the immune complexes were precipitated by the addition of protein A-Sepharose beads

(Amersham Biosciences). After washing in IP buffer, pellets or supernatants were separated by SDS-gel electrophoresis and analyzed by Western blotting.

Cell proliferation and anoikis assays

MCF-7 or MCF-7 SVV cells were analyzed for cell proliferation using an MTT assay in the presence of 1 mg/ml thiazolyl blue tetrazolium bromide (Sigma-Aldrich), and quantification of absorbance at 595 nm, as described (Dohi et al., 2007). For analysis of anoikis-associated apoptosis, MCF-7 or MCF-7 SVV cells were grown on ultralow attachment plates (Costar), harvested at increasing time intervals (d. 2-5) at 37°C, and analyzed for DNA content by propidium iodide staining and flow cytometry. Data were analyzed using FlowJo software, and the cellular fraction with hypodiploid, i.e. sub-G1, DNA content was quantified (Dohi et al., 2007). In some experiments, mitochondrial fractions were isolated from MCF-7 or MCF-7 SVV cells using a mitochondria isolation kit (Pierce, IL). Cytosol fractions or mitochondrial pellets were dissolved in lysis buffer and processed by Western blotting.

Adenoviral transduction

A replication deficient adenovirus (pAd) encoding wild type survivin (pAd-Survivin) or GFP has been described (Dohi et al., 2007). A replication-deficient adenovirus encoding a survivin T34A/C84A double negative mutant was obtained using the pAdEasy system. To construct the shuttle vector, a *Hind*III/*Xba*I fragment of pcDNA3.0-T34A/C84A generated by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) was inserted into pAdTrack-CMV to generate

pAd-survivin-T34A/C84A. The shuttle vector was linearized with *Pme*I, electroporated in *E.Coli* BJ5183 for homologous recombination, and colonies were selected in 50 μg/ml kanamycin. Each pAd construct (4-8 μg) was digested with *Pac*I, transfected in HEK293T cells by Lipofectamine, and cultures were analyzed for GFP expression, by fluorescence microscopy. High-titer viral stocks were generated by Adeno-X Virus Mini Purification Kit (Clontech), and the viral titer was quantified by green fluorescence forming units in HEK293T cells infected with serial dilution of the viral stock. Target cultures were transduced at a multiplicity of infection (moi) of 50 for 8 h at 37°C in complete medium, washed with PBS, pH 7.4, and replenished with complete medium. Cells were analyzed after additional 48 h at 37°C, and the transduction efficiency (>90% of the cell population) was estimated by GFP expression and fluorescence microscopy.

Promoter activity

Various cell types were transfected with an NFκB-responsive element fused to luciferase, or, alternatively, with a 1.9 kb fragment of the fibronectin promoter fused to luciferase (pGL-Fib1900), using Lipofectamine 2000. A total of 4ug of DNA was used and after 24 h at 37°C, cultures were harvested, and analyzed for β-galactosidase-normalized luciferase activity in a luminometer. In some experiments, MCF-7 SVV cells were transfected with control, TAB1- or TAK1-directed siRNA, and analyzed for NFκB luciferase promoter activity after 24 h.

Cell migration and invasion assays

Analysis of cell migration was carried out using 6.5-mm Transwell chambers (8-μm pore size; Costar). Inserts were prepared by coating the upper and lower surfaces with 15 μg/ml collagen (Cohesion, Palo Alto, CA) for 18 h at 4°C, followed by a blocking step with DMEM containing 0.25% heat-inactivated BSA for 1 h at 37°C. The various cell types were harvested, suspended in DMEM containing 0.25% heat-inactivated BSA, and added ($1x10^5$) to the upper chamber, with aliquots of conditioned medium collected from NIH3T3 fibroblasts placed in the lower chamber as chemoattractant. After a 1 h incubation, non-migrating cells were removed mechanically from the upper chamber using a cotton swab. Cells migrated to the lower surface of the Transwell membrane were fixed in methanol for 10 min at 22°C, and membranes were mounted on glass slides using Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). Cell migration was quantified by counting the number of stained nuclei in five individual fields in each transwell membrane, by fluorescence microscopy, in duplicate.

For analysis of cell invasion, the upper transwell chamber (8-μm pore size; Costar) was coated with 0.5 μg Matrigel (Collaborative Research, Bedford, MA) diluted in cold water, and allowed to air dry. After incubation with DMEM for 1 h, the various cell types ($1x10^5$) were added to the upper chamber for 6-24 h at 37°C. Cells that had invaded the lower surface of the membrane were fixed with methanol, stained with DAPI, and quantified by fluorescence microscopy. In some experiments, cells were incubated with the following pharmacologic inhibitors of Src (PP-2, 50 μM; SU6656, 25-

50 μM), MEK (PD98059, 50 μM, or U0126, 25 μM), PI3 kinase (LY290042, 50 μM), or vehicle (DMSO) for 1 h at 37°C, added to Matrigel-coated membranes, and analyzed for cell invasion by fluorescence microscopy.

Recombinant protein expression

cDNA constructs encoding recombinant full-length survivin (residues 1-142), full-length XIAP, Val80Asp XIAP mutant (V80D) (Lu et al., 2007), or full length IκBα RING-less XIAP (RING-Δ, residues 1-442), or E3 ligase-deficient XIAP His467Ala mutant(Addgene) were amplified by PCR, confirmed by DNA sequencing and cloned into the pGEX-4T1 expression vector without or with an HA-tag epitope. The various constructs were expressed as GST fusion proteins in BL-21 *E.Coli,* with removal of the GST frame by thrombin cleavage for 1 h at 37°C, followed by neutralization with benzamidine.

Electrophoretic Mobility Shift Assay (EMSA)

Twenty μg of nuclear extracts prepared from wild type or $XIAP^{-/-}$ MEF stably transfected with survivin were incubated without or with TNFα in the presence of ^{32}P γ-labeled 45-mer double-stranded NFκB oligonucleotide derived from the human immunodeficiency virus long terminal repeat, 5'-CGCTGGGGACTTTCCAGGGAGGCGTGG-3'. Incubations were carried out in buffer containing 10 mM Tris HCl, pH 8.0, 150 mM KCl, 0.5 mM EDTA, 0.1 % Triton-X 100, 12.5 % glycerol (v/v), 0.2 mM DTT for 1 h at 22°C. Poly-dIdC (Sigma) and sonicated salmon sperm DNA (Stratagene) were added to block non-specific binding. DNA-

protein complexes were separated by electrophoresis on 5% native polyacrylamide gels, and radioactive bands were visualized by autoradiography. A double-stranded ^{32}P γ-labeled mutant oligonucleotide, 5'-TTGTTACAACTCACTTTCCGCTGCTCACTTTCCAGGGAGGCGTGG-3', or wild type unlabeled oligonucleotide was used in competition experiments. In some experiments, DNA-protein complexes were incubated with an antibody to p65 NFκB for 1 h at 22°C, and samples were analyzed by autoradiography.

Modulation of IκBα phosphorylation

Recombinant IκBα (0.5 μg) was mixed with extracts of XIAP$^{-/-}$ DLD1 cells after transfection with control siRNA or TAB1-directed siRNA (5 μg) in the presence of recombinant XIAP, XIAP RING-Δ, XIAP V80D or H467A mutant (1 μg) in buffer containing 40 mM Tris, pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, 50 mM ATP for 20 min 30°C. Modulation of IκBα phosphorylation under the various conditions tested was analyzed with a phospho-IκBα antibody by Western blotting, and quantified by densitometry. For some IκBα phosphorylation studies, cells were incubated with proteasome inhibitor, lactacystin (1μM, Calbiochem, Cat # 426100).

***In vivo*metastasis model.**

All experiments involving animals were approved by an Institutional Animal Care and Use Committee. Female SCID/beige mice (6-8 wk of age) were anesthetized with ketamine hydrochloride, the abdominal cavity was exposed by laparotomy, and animals were injected in the spleen with parental MCF-7 or MCF-7 SVV cells ($2x10^6$) stably

transfected with a luciferase cDNA. To avoid potential confounding effects on metastasis by the continuous growth of a primary tumor, the spleen was removed 24 h after injection of the tumor cells. The incision was closed in two layers with vicryl 5/0 and wound clips. On d 1, 3, 7, and 11 after injection, animals were analyzed for metastatic dissemination to the liver by bioluminescence imaging using an IVIS-100 camera system for detection of luciferase expression (Xenogen, Alameda, CA). Briefly, mice were anesthetized with isoflurane and intraperitoneally injected with 2.2 mg luciferin sodium salt (GOLD Bio Technology, Inc) in PBS, pH 7.4. During image acquisition, isoflurane anesthesia was maintained using a nose cone delivery system. Both supine and prone images were scanned for a 3 min acquisition interval. Each image was acquired sequentially three to four times, and data were collected at the time of peak luminescence. The bioluminescence images were overlaid on black and white photographs of the mice collected at the same time. Signal intensity was quantified as the sum of all detected photon counts within a region of interest using Living image software (Xenogen, version 2.50). On d 11, all mice in the two groups were sacrificed and their livers were resected, and analyzed by bioluminescence, *ex vivo*. In some experiments, wild type ($XIAP^{+/+}$) HCT116, $XIAP^{-/-}$ HCT116 (five animals/group), wild type INS-1 (4 animals), or INS-1 stably transfected with survivin (6 animals) or Bcl-2 (five animals) were injected ($5x10^6$ cells) in the spleen of SCID/beige mice, followed by splenectomy as described above. Mice were monitored for blood glucose content twice weekly, and sacrificed after 3 weeks.

Histology

Livers from the various animal groups reconstituted as above were fixed in buffered formalin, and embedded in paraffin. For insulin staining, sections were deparaffinized, rehydrated in water, and quenched for endogenous peroxidase. Epitope heat retrieval was carried out by steaming the slides in 10% sodium citrate for 20 min. Processed slides were rinsed in PBS, pH 7.4, and stained with an antibody to insulin using standard avidin-biotin-peroxidase technique (Histostain-plus, Zymed Laboratories). Slides were incubated with DAB as a chromogen and counterstained with hematoxylin. Control sections were processed as above with non binding IgG, and resulted in no detectable staining.

Statistical analysis

Data were analyzed using the unpaired t test on a GraphPad software package for Windows (Prism 4.0). A p-value of 0.05 was considered as statistically significant.

Chapter 6: Final thoughts and Future directions

Tumor metastasis, the leading cause of 90% of cancer deaths is one of the greatest unresolved mysteries of cancer research (Gupta and Massague, 2006). Research over the past several years has helped us in understanding the molecular mechanisms involved in tumor progression although metastasis, being so complex, still remains a 'big black box'(Eccles and Welch, 2007). There are no therapies currently available that focus primarily on the metastatic aspect of the tumors. Classical treatments for tumor metastasis are similar to the therapies used to target primary tumors which are radiation therapy or chemotherapy (Steeg, 2006). In an era of targeted therapy which is based on molecular mechanisms we need to understand new aspects in order to get a better understanding of the metastatic cascade so as to devise novel therapeutic strategies to fight metastasis. Gene expression profiling of the metastatic tumors has helped us in identifying interesting targets and provided useful insights about the regulation of different steps of metastasis (Ramaswamy et al., 2003). Some of these studies have clearly demonstrated the involvement of apoptosis regulators in tumor progression (Um et al., 2004) . For example, on comparing various human or mouse cancer cell lines, an inverse correlation has been demonstrated between apoptotic sensitivity and the metastatic ability of the cells when injected into nude mice (Glinsky et al., 1997). Hence, the classical view is that apoptosis regulation is one of the key regulatory mechanisms required by tumor cells to form distant metastasis.

For my thesis research, I have demonstrated that some of the anti-apoptotic proteins particularly survivin play a bigger and broader role in metastasis by upregulating a broad gene expression program leading to the upregulation of certain cell adhesion molecules. We have shown that survivin mediated gene regulation requires intermolecular co-operation between survivin and its related cofactor, XIAP leading to activation of NFκB pathway. Although a role of XIAP in NFκB activation has been recognized previously (Srinivasula and Ashwell, 2008), our studies have identified a novel requirement of survivin in this pathway. We have dissected the structural requirements of survivin-XIAP mediated NFκB activation and demonstrated that synergistic cooperation of survivin and XIAP leads to enhanced IκB-α phosphorylation (Figure 3-3A), a prerequisite for NFκB activation. This pathway depends on the integrity of TAB1-TAK1 axis (Lu et al., 2007) for phosphorylation of Iκbα and stimulation of NFκB promoter activity *in vivo*. Once NFκB is activated, it leads to upregulation of several target genes, one of which is fibronectin which has been identified by this study and other studies (Lee et al., 2002) to be a bona fide NFκB target. Activation of fibronectin gene expression initiates the outside-in signaling by engaging with their cognate receptors, integrins. This leads to the activation of cell motility kinases, focal adhesion kinase (FAK) and Src kinases, the downstream mediators of IAP mediated tumor invasion and metastasis. Taken together our study has confirmed the importance of IAPs in tumor dissemination and dissected the upstream and downstream requirements of IAP mediated metastasis (Figure 6-1).

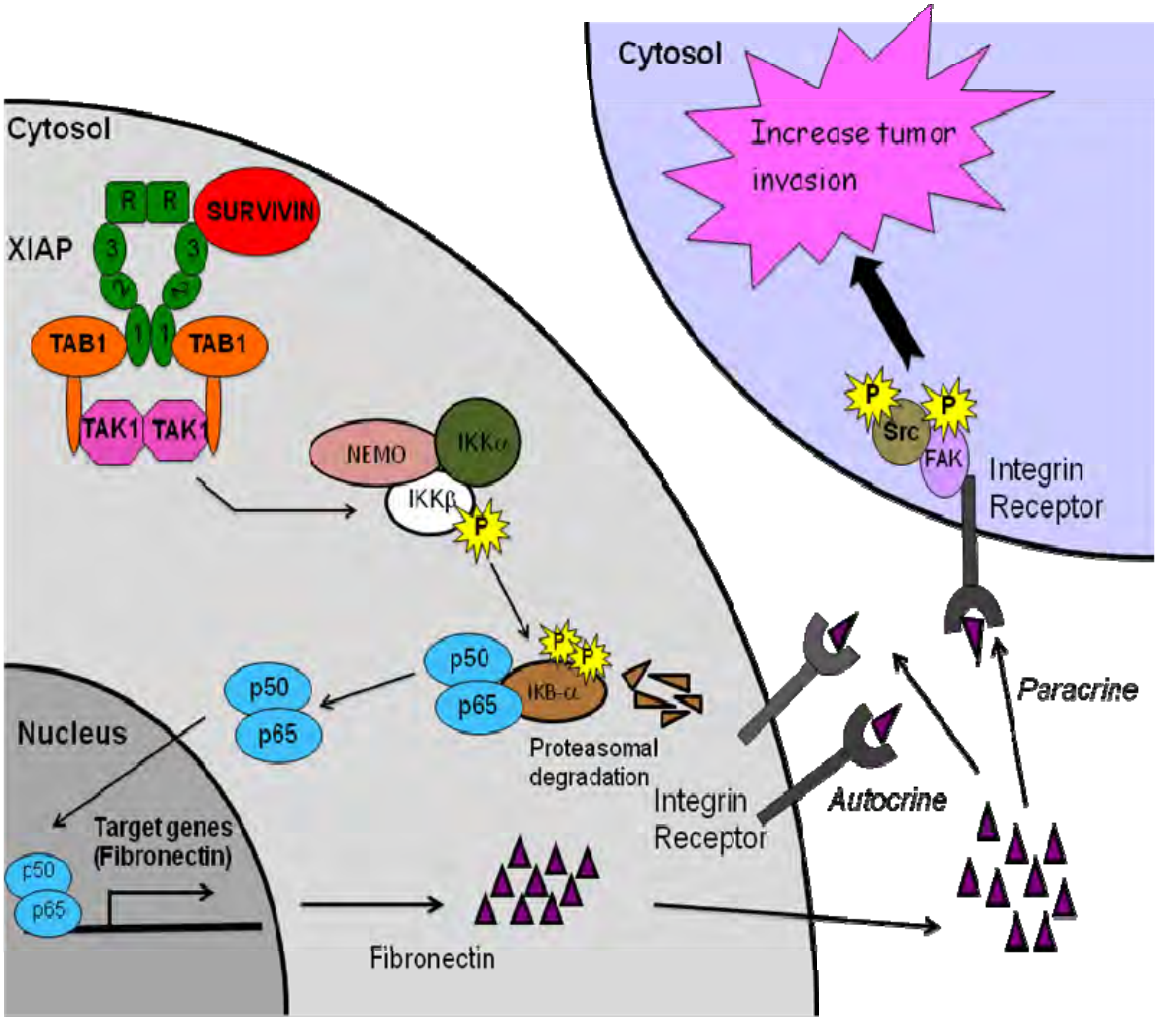

Figure 6-1: Model for IAP mediated tumor invasion

Survivin has been shown to function as an upstream regulator of gene expression. Survivin, a unique member of the IAP family mediates many diverse roles ranging from cell death inhibition, mitotic regulation and cell cycle checkpoint surveillance (Altieri, 2006). Due to its involvement in multiple signaling pathways involved in tumor maintenance, it has been rightly mentioned by Altieri *et. al.* (Altieri, 2008) as a classical nodal protein which can be utilized for targeted drug discovery. The main networks described are cell division and the cell death networks (Altieri, 2008). Our data adds a completely novel component to the existing networks of survivin and demonstrates for the first time the mechanistic role of survivin in the transcriptional control of cell adhesion molecules by activation of a broad transcription factor, NFκB (Figure 6-2).

Because of the diverse roles of survivin, it is difficult to say which function of survivin is playing an important role in tumor progression and metastasis *in vivo*. In our analysis, we have shown that metastatic role of survivin is independent of its anti-apoptotic function as we observe significant increase in invasion in Ins-1 cells overexpressing survivin (Ins-SVV) *in vitro* (Figure 2-5 B) and extensive metastasis *in vivo* (Figure 4-3) . Ins-SVV cells does not inhibit apoptosis due to defective mitochondrial import system (Dohi et al., 2007). Similar paradigm is applied to XIAP, as disruption of its anti-apoptotic function by transfecting XIAP Asp143Ala/Trp310Ala double mutant (D143A/W310A) that does not bind caspase 3 (D143A mutation), nor caspase 9 (W310A mutation) in a $XIAP^{-/-}$ HCT116 cells (Lewis et al., 2004) shows significant invasion (Figure 2-5D). This is suggestive that the anti-apoptotic function of IAPs is not essential for promoting metastasis although we still need further evidence *in*

vivo to solidify the findings and differentiate the anti-apoptotic functions and metastatic functions of IAPs. Survivin has also been extensively studied as a mitotic gene and shown to be a part of the chromosome passenger complex (Adams et al., 2001). Our data does not address if the mitotic function is essential in survivin-mediated tumor invasion and metastasis. This can be addressed in several ways one of which may be using a dominant negative Survivin mutant (Surv-DD70, 71AA) that has been reported to disrupt the interaction of Survivin with Aurora B, resulting in failed cytokinesis and cell division defects (Cao et al., 2006). It will be interesting to explore if the cells carrying this mutant are able to invade and metastasize.

Moreover, in this study we have looked for the role of survivin in metastasis with respect to tumor invasion but failed to look at the migratory and cytoskeletal changes that are potentially involved and important in IAP mediated metastasis. We performed a phalloidin staining for actin cytoskeleton in MCF-7 SVV cells and MCF-7 cells and did not observe any significant changes (Figure 2-1E). However, a better approach would be to perform a time lapse study on IAP overexpressing cells in order to better understand the changes in actin modifications. Additionally, studying the changes in the activity of some of the proteins important in cell migration like Rho and Rac in IAP overexpressing cells will be useful to strengthen our observations.

The current study utilizes the intrasplenic injection model to test the IAP mediated tumor metastasis *in vivo*. In this model, the spleen is resected after 24hrs of injection and liver metastasis is monitored for a period of 14-21 days. This model has given us promising results but we cannot overlook the potential pitfalls of this model system.

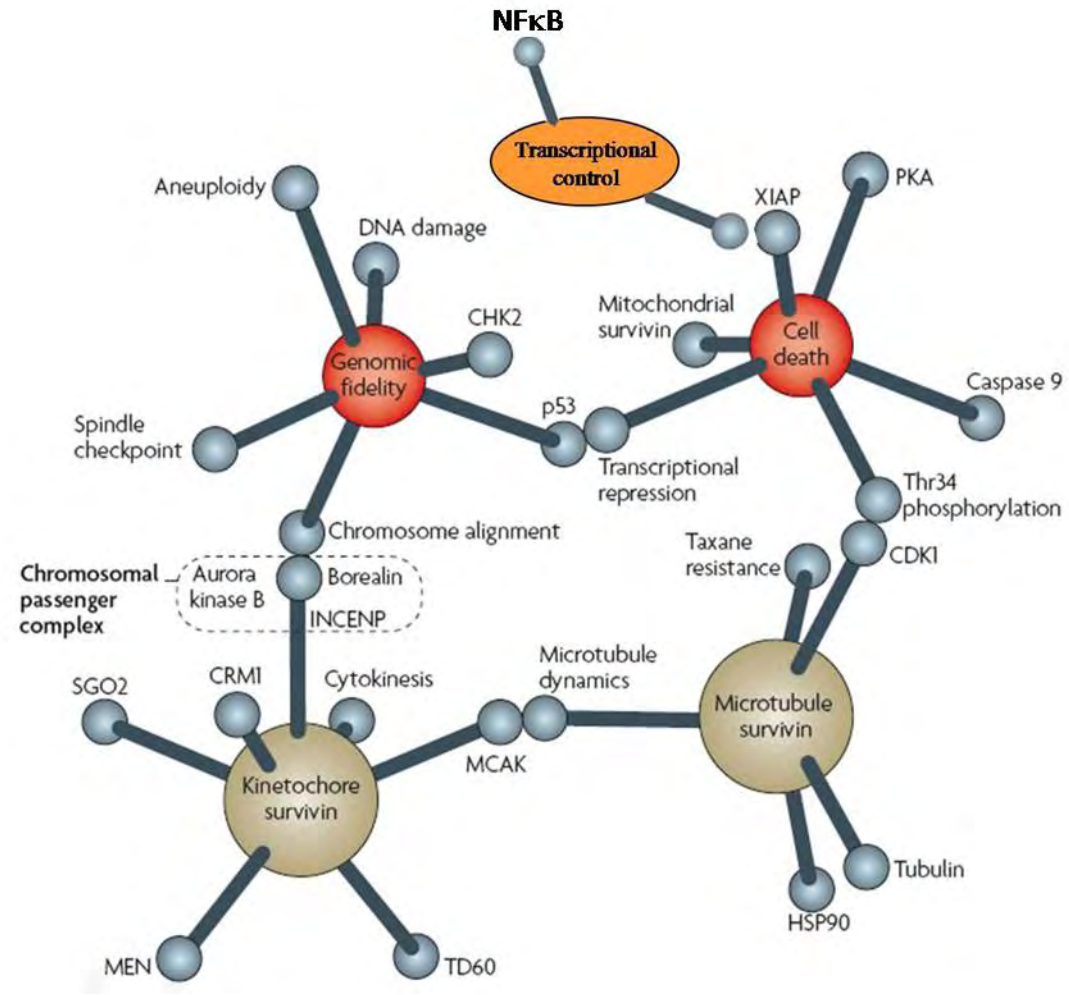

Figure 6-2: Connectivity map showing various diverse function of survivin

As shown in this map, survivin functions in cell death, mitotic regulation, checkpoint point regulation, subcellular trafficking. In addition to these, the work described in this thesis describes a new role of survivin in transcriptional control of transcription factor, NFκB. CDK1, cyclin-dependent kinase 1; CRM1, chromosome region maintenance protein 1; HSP90, heat shock protein 90; INCENP, inner centromere protein antigens; MCAK, mitotic centromere-associated kinesin; MEN, mitotic exit network; PKA, protein kinase A; SGO2, shugoshin 2. This Figure is modified from (Altieri, 2008).

Firstly, since spleen is a highly vascular organ and is rich in sinusoids (Cesta, 2006), it is highly likely that the cells are injected into the sinusoids of the spleen and hence enter into the circulation immediately thus bypassing the initial steps of metastasis. We have not ruled out this possibility but our initial experiments done for protocol approval from IACUC (Institutional Animal Care and Use Committee) have shown that resecting spleen after 1 hour of injection does not result in liver metastasis (data not shown) suggesting that not all cells have escaped into the circulation or the few cells that have escaped are not viable. We lack the data showing the viability of the transfected cells in the spleen. It is possible that the differences in tumor metastasis are arising because of differences in the viability of these cells in spleen. However, we have investigated the proliferation kinetics of cells injected into the spleen (Fig. 2-1) and we did not observe any significant changes but it will be useful to know if they show similar kinetics *in vivo*. Additionally, we have performed bioluminescence imaging of mouse injected with MCF-7 and MCF-7 SVV cells after 24hrs of injection and immediately before resection and we did not observe any changes in the cell viability (Figure 6- 3). However, to further confirm our findings, it will be useful to isolate the cells from resected spleens and perform cell viability assays *in vitro*.

Secondly, in our intrasplenic injection model system, we resected the spleens in order to remove the primary tumor so as to allow for an unbiased evaluation of metastatic dissemination, not confounded by the growth of a primary tumor. It was done to avoid the complications arising due to large primary tumor masses as sometimes the primary tumor burden overwhelms the animal much before the appearance of macroscopic

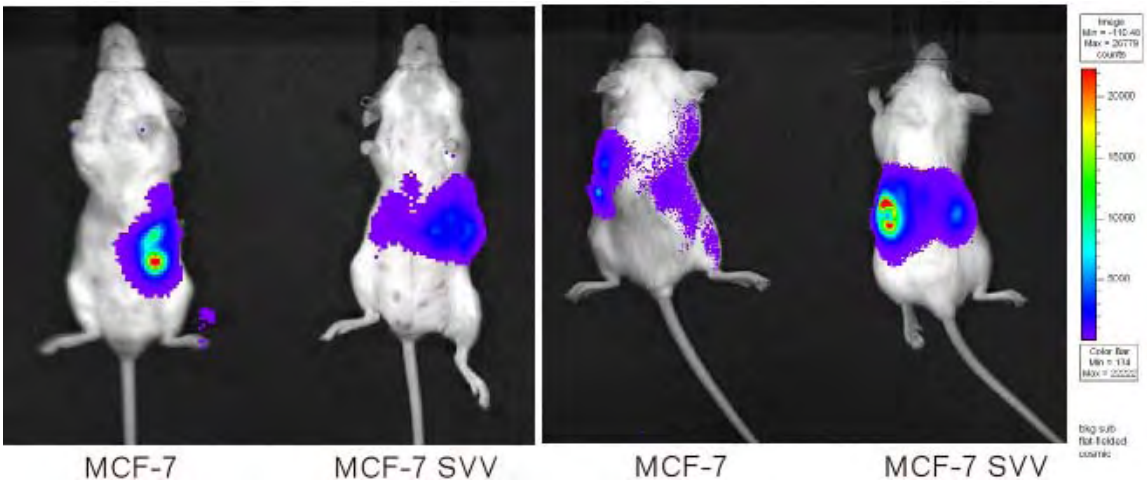

Figure 6-3: Intrasplenic injection of survivin-over expressing cells

MCF-7 or MCF-7 SVV cells stably transfected with a luciferase cDNA.were analyzed by bioluminescence imaging 24hrs after injection and immediately before splenectomy. Ventral view (left) and dorsal view (right)

metastasis. Moreover, in some cases, primary tumor size strongly correlates with the likelihood of forming metastasis (Price et al., 1990; Safarians et al., 1996). However, interestingly, there are some studies that have shown a rapid outgrowth of metastasis after removal of primary tumor (Peeters et al., 2006). It has been shown that resection of primary tumors leads to loss of angiogenesis inhibition as primary tumors produce angiogenic and other growth factors important for growth of metastatic tumors (Li et al., 2001b). Several clinical studies have supported the concept of metastatic dormancy imposed by the primary tumors (Fisher et al., 1983; Peeters et al., 2008). This is particularly important in the context of this study and can be a possibility. However, most of these studies have looked at the influence of primary tumor on the 'growth of the metastatic tumor' at the secondary sites whereas this study was focused more on understanding the initial steps of metastasis which include invasion, intravasation, survival in circulation and extravasation. It is possible that on reaching the metastatic site, survivin overexpressing cells will survive and proliferate more at the distant sites due to their anti-apoptotic and mitotic effect but this has not been extensively looked at in the present study.

In our analysis, using gene silencing and functional blocking experiments, we have shown the importance of fibronectin in mediating tumor invasion in IAP expressing cells. This has physiological relevance as fibronectin has been extensively shown to be upregulated in a variety of tumors. A study by Gaggioli et. al (Gaggioli et al., 2007) showed that tumor derived fibronectin plays an important role in melanoma cell invasion. Additionally, gene expression profiling analysis has clearly shown a positive correlation

between enhanced fibronectin expression and acquisition of oncogenic and metastatic potential (Bittner et al., 2000; Clark et al., 2000). We have supporting evidence suggesting that fibronectin is one of the players in IAP-mediated tumor invasion. However, we lack the data that shows that exogenous presence of fibronectin is important for making a non-invasive cell type more invasive and metastatic. Based on our results, we believe that fibronectin is only one of the players and there are other parallel or converging pathways that might be important in IAP mediated tumor invasion, as survivin knockdown completely abolishes the ability of survivin overexpressing cells to invade (Figure 2-2 B) whereas in the same cell line, fibronectin silencing results only in 50% reduction (Figure 2-9 B).

Fibronectin, a high molecular glycoprotein is highly polymorphic and is known to exist in 20 different isoforms due to the alternate splicing of the primary transcript (Kornblihtt et al., 1984). The cellular fibronectin molecule has three regions that can be alternately spliced- EDA (extra domain A), EDB (extra domain B) and IIICS (type III homology connecting segment). Several studies have reported a differential pattern of alternately spliced isoforms in cancer tissues (Carnemolla et al., 1989; Oyama et al., 1993). However, this study does not address whether IAPs modulate the expression of a particular isoform of fibronectin and which isoform of fibronectin is important for IAP mediated tumor invasion. A more detailed characterization of different isoforms of fibronectin in IAP expressing cells will be useful in resolving the issue.

Moreover, in this study we have only looked at one of the cell adhesion molecules, fibronectin. Our data suggests a significant modulation of other ECM

components like collagens in IAP overexpressing cells (Figure 2-6A) although it has not been extensively looked at in the present study. Some of the cell adhesion molecules like VCAM-1(van de Stolpe et al., 1994), ICAM-1 (Iademarco et al., 1992), MMPs (He, 1996) have also been shown to be NFκB targets hence further work needs to be performed to dissect the importance of other ECM components in mediating metastatic dissemination in IAP expressing cells.

In our analysis, we have demonstrated that NFκB activation in IAP expressing cells leads to upregulation of fibronectin gene expression. A role of fibronectin as an NFκB responsive gene has been controversial in the only two papers that appear in literature (Lee et al., 2000; Lee et al., 2002). Our data strongly supports a positive regulation of fibronectin by NFκB using two independent approaches, reduction of fibronectin protein expression after siRNA targeting of the p65 subunit (Figure 3-2B) and enhanced expression of fibronectin after NFκB activation by TNFα (Fig. 3–2C), one of the known activators of the NFκB pathway. However, both these results show a modulation of fibronectin by activated NFκB only at the 'protein level' hence we require more direct evidence of fibronectin regulation by NFκB at the transcriptional level in survivin over-expressing cells in order to confirm our findings.

In the context of this study, there is growing evidence for a role of NFκB signaling in metastasis, largely associated with stimulation of epithelial-mesenchymal transition (EMT), expression of matrix metalloproteinase-9 (MMP-9), and repression of putative metastasis-suppressor genes, *in vivo* (Naugler and Karin, 2008). In our experimental system, IAP-induced NFκB activation did not result in morphological

features of EMT, as judged by comparable expression of E-cadherin in MCF-7 or MCF-7 SVV cells, or changes in MMP-2 or -9 levels. However, there is accumulating evidence that some of the transcription factors best characterized for their role in EMT like Snail (Nieto et al., 1994) can drive tumor progression in the absence of EMT primarily by enhancing cell adhesion and migration (Barrallo-Gimeno and Nieto, 2005). Snail expression has been detected in an increasing number of carcinomas and associated with invasive ductal breast carcinomas (Blanco et al., 2002)and hepatocarcinomas (Sugimachi et al., 2003). A recent study (Haraguchi et al., 2008) showed that Snail regulates the expression of various ECM components which leads to enhanced cell migration and re-attachment at the secondary site. This is particularly important in context to this study as IAP overexpressing show constitutive activation of NFκB and increased expression of fibronectin gene expression and Snail has been previously reported to be upregulated by NFκb activation (Julien et al., 2007). Hence, it will be interesting to explore if Snail is involved in mediating this program in IAP expressing cells.

Fibronectin binds to their surface receptors integrins and leads to the phosphorylation and activation of cell motility kinases, FAK and Src. Integrin signaling has been shown to cross talk with the growth factor signaling. Emerging evidence show that growth factor mediated effects on cell proliferation, migration and adhesion depend on specific integrins (Eliceiri, 2001). Conversely, integrin mediated cell migration and invasion require growth factor stimulation. For example, αvβ5 bearing melanoma cells require IGF-1 pre-stimulation for metastasis (Brooks et al., 1997). Similarly other studies have shown a dependence of integrins on growth factor signaling to mediate the

downstream effects. It is possible that growth factor activation can be one of the 'other converging pathways' that might play an important role in tumor invasion of these IAP expressing cells. In support of this hypothesis, we have some preliminary data showing high expression of Epidermal Growth Factor receptor (EGFR) in breast cancer cell line overexpressing survivin (MCF-7 SVV). Additional experiments need to be performed to confirm the findings.

Constitutive activation of NFκB has been shown in both solid tumors and hematological malignancies and hence it has been extensively targeted for anticancer therapy. Since NF-κB functions as an essential factor promoting cell survival of normal cells during stress and immune responses, it is logical to expect toxicity effects of NFκB inhibitors, which cannot be tolerated when designing effective cancer therapeutics. However, we have shown an 'alternate' pathway for activation of NFκB which requires survivin. Since survivin show differential expression in tumor cells as compared to normal cells, targeting survivin can be used as a powerful approach for specifically targeting NFκB activity in tumor cells. The major challenge that can be conceptualized when using this approach is the identification of NFκB driven tumors. However, advanced genomic technology has been successful in identifying a subset of diffuse large B-cell lymphoma (DLBCL) on the basis of NFκB gene signature and preclinical studies have successfully validated NFκB as a therapeutic target in these lymphoma subtypes (Davis and Staudt, 2002). Hence, we can utilize the approach of gene expression profiling to identify other aggressive tumors that are NFκB-driven so that we can use survivin antagonists in a more meaningful manner. Even though survivin is not a surface protein

and lack catalytic activity, two of the important qualifications to be a ‘good therapeutic target’, several survivin antagonists tested in clinical trials have shown promising results. Some of these antagonists like YM155 are in advanced phase of clinical trials and we believe, with the present findings of survivin’s role upstream of gene expression, these antagonists can have broader clinical implications in both early and late stages of tumor progression.

We have demonstrated that FAK and Src motility kinases are key mediators tumor metastasis in IAP expressing cells. Disruption of FAK activity using dominant negative mutant, FRNK and inhibition of Src phosphorylation using pharmacological inhibitorslike PP2 and SU6656 dramatically reduces the IAP mediated tumor invasion (Figure 2-12B, C). Additionally we have shown that phosphorylation and activation of Src at Tyr-416 in insulinoma cells (Figure 2-11E). IAP mediated Src activation can also be exploited for better targeting of metastatic tumors. Some of the commonly used Src inhibitors that are approved for treatment against chronic myelogenous leukemia (CML) and Acute Lymphoblastic leukemia (ALL) are Dasatinib (Bristol Meyers Squibb Oncology, Princeton, NY) , AZDSKI-606 (AstraZeneca, Macclesfield, United Kingdom) and SKI606 (Wyeth Research, Pearl River, NY). They are also being tested for other solid tumors. Under *in vitro* conditions, all of these have been shown to suppress tumor invasion. Dasatinib has been shown to suppress invasion in head and neck squamous cell carcinomas (Johnson et al., 2005) and prevent the formation of liver metastasis in orthotopic mouse model of pancreatic carcinoma (Trevino et al., 2006). AZD0530 has been shown to inhibit invasive ability.of tamoxifen breast cancer cells (Hiscox et al.,

2006). Similarly SKI-606 has also been shown to inhibit tumor extravasation (Weis et al., 2004). Even though all these studies show an important role of Src in the initial steps of metastasis, these Src inhibitors have always been tested when metastatic tumors have already been established. Based on our data, we believe that the Src inhibitors will be more effective as ‘adjuvant therapy ‘especially after the surgical resection of the primary tumor as it is possible that some of the tumor cells are leaked into the circulation during the surgical operation. Hence we can prevent the initiation of metastasis by treating these patients with Src inhibitors immediately after the surgical resection of the primary tumor.

To conclude, the study presented in the thesis has both mechanistic insights and clinical implications. This work for the first time places survivin upstream of gene expression and unravels some of the essential components of IAP mediated metastasis which can be utilized for more targeted and effective anti-metastatic therapies among the others that are already being or intended to be investigated in future.

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